AN ULTRASTRUCTURAL AND CYTOCHEMICAL INVESTIGATION OF ENDOMETRIUM FROM PREGNANT AND NONPREGNANT GILTS

BY

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I dedicate this dissertation to my parents, Harrell and Clara Renegar, who instilled within me the values needed to succeed in any endeavor, and to my wife, Marilyn, who patiently and lovingly provided the support necessary for completion of this effort.

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Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

AN ULTRASTRUCTURAL AND CYTOCHEMICAL INVESTIGATION OF ENDOMETRIUM FROM PREGNANT AND NONPREGNANT GILTS

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Studies were designed to examine cellular mechanisms that regulate direction of release of uterine secretory products in swine during the period when chemical messages from blastocysts signal the maternal unit that pregnancy is establsihed (maternal pregnancy recognition) (Days 12 to 15 postestrus). Uterine endometrium taken from gilts during early pregnancy and diestrus was examined by light and electron microscopic and biochemical techniques to examine effects of pregnancy.

Changes in morphology of glandular epithelial tight junctions and basement membranes in pregnant and nonpregnant gilts were not associated with maternal pregnancy recognition. Basement membrane thickness increased between Days 10 and 12 of gestation and the estrous cycle. Changes in endometrial collagenase-like activity, total collagen and free hydroxyproline, which may reflect modification of basement membrane structure, were not associated with maternal pregnancy recognition. Direction of release of uterine secretory products by the glandular epithelium did not change during the period studied; however, secretory

activity decreased on Day 18 in nonpregnant gilts. Cellular mechanisms regulating direction of release of uterine secretory products in swine were not found to be associated with changes in epithelial cell ultrastructure. Data demonstrated blastocyst influence on uterine morphology and secretory activity.

Studies were designed to examine placental uteroferrin (Uf) transport and its distribution in fetal pigs. Uteroferrin is the major placental iron transport protein in pigs. Peroxidase-antiperoxidase (PAP) localization of Uf demonstrated uptake by epithelial cells of chorioallantoic areolae and subsequent release into placental capillaries. Uteroferrin concentrations in umbilical vein blood were greater (84%) than in umbilical artery blood. Uteroferrin was localized in Day 75 fetal kidney tissue by the PAP technique, and its presence in fetal urine was demonstrated by acid phosphatase activity, two-dimensional polyacrylamide gel electrophoresis and immunodiffusion analysis. Isolated Day 75 fetal liver plasma membranes bound ^{125}I-Uf in a dose-dependent manner. Binding, determined by light and electron microscope autoradiography, was specific for reticuloendothelial cells lining liver sinusoids. Uptake of bound Uf was mediated by coated pit formation. A mechanism was determined for direct iron transport to fetal liver, a major site of hematopoiesis, and to the allantoic sac for storage.

CHAPTER I
INTRODUCTION

Recurring reproductive cycles characterized by periods of sexual receptivity (estrus) and nonreceptivity (diestrus) are found in most domestic mammals. These widely varied behavior patterns are controlled for the most part by hormonal secretions of the ovary. Estrogens produced by developing follicles around the time of ovulation are responsible for sexual receptivity. The period of sexual nonreceptivity is controlled by progesterone produced by the ovarian corpus luteum (CL) which develops from granulosa cells associated with newly ovulated follicles. Progesterone not only influences behavioral aspects of the reproductive cycle, but also stimulates the uterine endometrium to develop and to synthesize and secrete a variety of molecules into the uterine lumen to serve as nutrients for the developing conceptus. These secreted nutrients (histotroph) are especially important in early development of large domestic species since attachment of the developing conceptus to the uterus occurs several weeks after ovulation and fertilization of ova.

In the case of a nonfertile mating or early embryonic death the CL has a limited lifespan that is determined by the uterus. In nonpregnant females of the domestic horse, cow, pig and sheep, the uterus synthesizes and releases prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) into the uterine vasculature, and $PGF_{2\alpha}$ acts at the ovary to cause regression of the CL (reviewed by Bazer et al., 1981). Cessation of endometrial synthesis and reabsorption of uterine secretory products accompanies CL regression. Corpus luteum

regression is followed by follicle growth and a new period of sexual receptivity which allows another opportunity for conception. In pregnant animals the conceptus "signals" its presence at an appropriate time to prevent movement of uterine $PGF_{2\alpha}$ into the circulation in sufficient quantities to cause CL regression. Response of the uterus to an embryonic signal is decreased $PGF_{2\alpha}$ release into the circulation and continued synthesis of histotroph, and this response is referred to as "maternal recognition of pregnancy."

In pigs the embryonic signal is estrogen secreted by the developing blastocyst (Perry et al., 1973; Fischer, 1981), and estrogen is believed to act locally at the endometrium to prevent release of $PGF_{2\alpha}$ into the uterine vasculature. Bazer and Thatcher (1977) proposed a theory of maternal recognition of pregnancy in swine based on the local action of estrogen. These authors proposed that, in nonpregnant females, progesterone enhances $PGF_{2\alpha}$ production and movement into the uterine venous drainage (endocrine release). Ultimately $PGF_{2\alpha}$ reaches the CL to cause luteolysis. In pregnant females $PGF_{2\alpha}$ synthesis is also enhanced by progesterone, but estrogen of blastocyst origin directs release of $PGF_{2\alpha}$ into the uterine lumen (exocrine release). In this manner $PGF_{2\alpha}$ is prevented from exerting a luteolytic effect and synthesis of histotroph continues. The cellular mechanism that responds to estrogen by controlling the direction of $PGF_{2\alpha}$ release is not known. Bazer and Thatcher (1977) suggested that changes in permeability of the basement membrane of uterine glandular and surface epithelium may be involved. A portion of this dissertation research was designed to investigate cellular mechanisms involved in maternal recognition of pregnancy in swine.

Maternal recognition of pregnancy assures continued histotroph secretion to support development of the fetus prior to and following placental development. In horses, pigs, cows and sheep placental development proceeds in a noninvasive manner, and attachment to the uterus is achieved by interdigitation of microvilli on the placental and uterine epithelium (epitheliochorial placenta). Macromolecules necessary for fetal development are obtained by placental uptake of uterine secretions. In pigs, secretions of uterine glands are taken up by cup-shaped structures (areolae) in adjacent placental tissue and subsequently transported to the fetus.

The second portion of this dissertation research was designed to examine transport of uteroferrin (Uf), an iron containing uterine secretory protein, to the fetus following development of the placental membranes. Mother-to-fetus transport of iron for hemoglobin biosynthesis is of primary importance for fetal development. Seal et al. (1972) reported that maternal plasma iron (transferrin bound) was very slowly transported to fetuses of animals with epitheliochorial placentae and concluded that another mechanism is utilized for iron transport in these species. Palludan et al. (1969) demonstrated that placental iron transport in pigs was achieved by secretion of iron containing protein(s) from the uterine glands and subsequent uptake of this protein(s) by placental areolae. Uteroferrin is an iron containing glycoprotein isolated from uterine flushings of gilts during diestrus (Chen et al., 1973). Uteroferrin is synthesized and released by epithelial cells of uterine glands and taken up by cells of the placental areolae. Recently, Ducsay et al. (1982) determined that Uf is the major placental iron transport protein in pigs. The fate of Uf after entering cells of the placental areolae

is not known. Chen et al. (1975) suggested that Uf crossed placental tissues and entered allantoic fluid, which is a site of high Uf concentrations (Bazer et al., 1975). Buhi et al. (1982a) demonstrated that Uf lost its iron to transferrin in allantoic fluid, and Ducsay et al. (1982) reported that transferrin in allantoic fluid was taken up by placental tissue for transport to the fetus. Placental transport and fetal distribution of Uf were examined in the second portion of this dissertation research to determine if Uf plays only an indirect role in placental iron transport to the fetus (loss of iron to transferrin) or if Uf may function to transport iron directly to sites of hematopoiesis in the fetus. Neonatal anemia in piglets is a management problem that is to date handled by iron injections. An understanding of the processes involved in placental iron transport may allow development of management practices during gestation that alleviate or ameliorate costly postpartum treatment of piglets with iron dextran.

CHAPTER II
REVIEW OF LITERATURE

Introduction

The domestic pig, Sus scrofa domesticus, has an efficient reproductive strategy and is capable of producing a large number of offspring over a short period of time. High ovulation rate and uterine capacity, as well as a relatively short interval from birth to puberty (6 to 8 months) are important factors in this reproductive strategy. The reproductive tract of the pig consists of the ovaries, oviducts, uterus, cervix, vagina and external genitalia which must function properly for development and birth of a viable neonate. In addition, acquisition of nutrients for successful fetal development involves a complex interaction of fetal and maternal tissues. The following review of literature will describe reproductive anatomy and physiology of the female pig, development, structure and function of fetal membranes during pregnancy and maternal pregnancy recognition. In addition, placental iron transport and its subsequent utilization by the fetal liver will be briefly discussed.

Reproductive Anatomy of the Female Pig

The following descriptions are from Anderson (1974), Hafez (1974) and Dziuk (1977). Information from additional sources is indicated.

The ovary of the pig serves two functions: (1) development and release of the mature ova, and (2) secretion of ovarian sex steroids

(estrogen and progesterone). Numerous follicles and corpora lutea on the surface of the ovary obscure underlying connective tissue and cause the porcine ovary to resemble a cluster of grapes. Two areas can be identified in a cross section of the ovary, the cortex which contains ova, follicles, corpora hemorrhagica, corpora lutea and corpora albicantia and the medulla which contains nerves, lymphatics and blood vessels which enter the ovary at the hilus. A portion of the broad ligament termed the mesovarium is attached to the ovary at the hilus and functions to suspend the ovary within the body cavity.

Formation of a mature follicle begins by differentiation of ovarian tissues adjacent to the ova and culminates with the appearance of a fluid filled structure containing the ovum. The wall of a mature follicle has three tissue layers: granulosa, theca interna and theca externa. Theca interna cells are active in production of estrogen precursors which are converted into active estrogens by granulosa cells. Growth of the follicle is regulated by follicle stimulating hormone (FSH) from the anterior pituitary. Immediately prior to ovulation, the follicle of the pig attains a diameter of 8 to 11 mm. Luteinizing hormone (LH), which is synthesized and released into the blood by the anterior pituitary, stimulates follicular changes which lead to ovulation. In addition, LH initiates differentiation of granulosa and theca interna cells into luteal cells.

Following ovulation the once fluid filled cavity of the follicle becomes filled with blood and is termed the corpus hemorrhagicum. Continued differentiation of the granulosa and theca interna cells leads to decreased estrogen production, but increased progesterone production by the developing corpus luteum (CL). Differentiation of these cells is

accompanied by cellular hypertrophy to ultimately fill the follicular cavity with luteal tissue. The final structure is the mature CL. The mature size of the CL within a single animal is variable, with some CL twice as large as their smaller contemporaries (Perry and Rowlands, 1982). These authors reported a mean CL weight of 420 mg for the gilts in their study, and Dziuk (1977) reported that mature CL of the estrous cycle average 10 to 12 mm in diameter. Luteal tissue synthesizes and secretes progesterone during the luteal phase of the estrous cycle and pregnancy. Progesterone is necessary for preparation and maintenance of a uterine luminal environment capable of supporting a developing conceptus. If conceptuses are not present in the uterus of the pig by Day 12, the animal is deemed not pregnant and the CL undergo regression. Regression is believed to be induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a hormone released into the blood by the nonpregnant uterus. During CL regression there is both rapid luteal cell degradation including vacuolation and cytoplasmic disorganization and connective tissue invasion of the luteal tissue. Progesterone secretion declines rapidly during luteal regression while plasma estradiol increases in conjunction with a new wave of developing follicles. The corpora albicantia are formed at completion of luteal regression by connective tissue invasion of the area once occupied by the CL. In pregnancy, the CL are maintained (See Maternal Recognition of Pregnancy).

The oviduct is a muscular tube suspended within the peritoneal cavity by the mesosalpinx, a peritoneal fold derived from the broad ligament. The portion of the oviduct adjacent to the ovary is called the infundibulum and is expanded to form a funnel-like structure. The infundibulum and its fringe of irregular processes, the fimbriae,

function to direct the ova into the oviduct. The remainder of the oviduct consists of the anterior ampullary and posterior isthmic portions, and fertilization of the ova occurs in the region of their junction.

The uterus of the pig is bicornuate with long (1 meter) uterine horns and a small uterine body. Histologically, the uterus consists of three tissue layers. The outer layer, the serosa, is continuous with the mesometrium which suspends the uterus within the peritoneal cavity and supports blood vessels and nerves supplying the uterus. The next layer is the myometrium which consists of an outer longitudinal and inner circular layer of smooth muscle. The inner layer of the uterus is the endometrium. The endometrium consists of an epithelium lining the uterine lumen and a subepithelial region of connective tissue. Branched, tubular glands are present in the subepithelial connective tissue of the endometrium and their lumina open onto the endometrial surface. Secretions of the uterine glands and surface epithelium nourish developing conceptuses during gestation, and synthesis and release of these substances are regulated by estrogen and progesterone of ovarian and/or placental origin.

The cervix is a sphincter-like structure at the posterior end of the uterus and is characterized by a thick connective tissue wall and constricted lumen. The primary functions of the cervix are to form a barrier to bacterial invasion of the uterus, as a site of sperm deposition in the pig and to permit expulsion of the fetus.

The vagina is an organ for copulation and the terminal passageway for expulsion of the fetus.

Reproductive Physiology of the Female Pig

Complete reviews of the reproductive physiology of the female pig are available (Anderson, 1974; Dziuk, 1977; Pond and Houpt, 1978); however, the following discussion will be limited to the more salient features of reproductive physiology.

The domestic pig is polyestrous and shows no seasonality in its breeding pattern. The estrous cycle averages 21 days (19-23) and may be divided into four phases: estrus, metestrus, diestrus and proestrus. Estrus is the period of sexual receptivity of the female to a boar and is approximately 40 to 60 hours long. During this discussion, the first day of estrus will be designated Day 0 of the estrous cycle. During estrus, the ovary contains 15 to 20 mature follicles which ovulate approximately 40 hours after the beginning of estrus. Ovulation is induced by a rapid increase in LH which reaches peak concentrations in the peripheral plasma at the onset of estrus (ovulatory surge of LH). During estrus, plasma estrogen, FSH, and progesterone concentrations are low and LH concentrations rapidly decrease. With the termination of sexual receptivity, the animal enters the metestrus phase of the estrous cycle. Metestrus is characterized by increasing plasma concentrations of progesterone from the developing CL and low estrogen, FSH and LH. Diestrus begins on Days 3 to 4 following completion of CL development. Progesterone secretion is maximal during diestrus while LH, FSH and estrogen concentrations in the plasma remain low. On Days 15 to 16 the CL begin to regress and progesterone secretion decreases rapidly. Prostaglandin $F_{2\alpha}$ of uterine origin is believed to cause luteal regression (see Maternal Recognition of Pregnancy). Proestrus begins as plasma

progesterone concentrations decrease. At the same time, estrogen concentrations increase as preovulatory follicles develop. Plasma estrogen concentrations reach a maximum on Day 18 and subsequently decline to low levels prior to the beginning of estrus. Near the end of proestrus, plasma LH concentrations begin to increase toward the ovulatory surge of LH.

Uterine Secretory Action During the Estrous Cycle and Early Pregnancy

General Quantitative and Qualitative Changes

Surface and glandular epithelium of porcine endometrium (Chen et al., 1975) secrete increased quantities of protein into the uterine lumen during diestrus (Murray et al., 1972). Failure of viable embryos to reach the uterus, as is the case during the estrous cycle or after a nonfertile mating, results in a rapid decline in protein secretion at the end of diestrus (Murray et al., 1972). If pregnancy is established, uterine secretory activity continues throughout gestation and these secretions (histotroph) are believed to supply metabolic precursors for growth and development of the conceptuses (Amoroso, 1952; Bazer, 1975). Following attachment of the pig blastocyst to the uterus, histotrophic nutrition is partially replaced by hemotrophic nutrition. Hemotrophic nutrition is defined as direct exchange of substances between maternal and fetal blood by diffusion through placental tissues (Grosser, 1927). Areolae, which are cup-shaped specializations of the chorion in contact with the mouth of uterine glands, are responsible for histotrophic nutrient uptake by the conceptus after placental development (Brambell, 1933; Wislocki and Dempsey, 1946; Chen et al., 1975).

Murray et al. (1972) examined quantitative and qualitative variations in the secretion of protein by the porcine uterus during the estrous cycle. Total recoverable protein in uterine flushings was low on Days 2 to 9, but began to increase on Day 10 reaching a peak on Day 15. After Day 15, total protein content of uterine flushings decreased sharply so that on Days 17, 18 and 20 values were comparable to values on Days 6 to 9. Sephadex G-200 profiles of uterine flushings from Day 6 to 9 indicated three distinct protein fractions (I-III) with molecular weights estimated at greater than 200,000, 200,000 and 90,000, respectively. On Days 10 to 16, during the time of increased protein secretion, two additional protein fractions (IV and V) appeared. Fraction IV had an estimated molecular weight of approximately 45,000 and was characteristically lavender in color. The molecular weight of Fraction V was approximately 20,000 (Murray et al., 1972). Squire et al. (1972) further characterized uterine protein secretions during the estrous cycle by polyacrylamide gel electrophoresis. Six acidic proteins and one major basic protein were present in Fractions V and IV, respectively.

Krishnan and Daniel (1967) isolated a protein called blastokinin from uterine secretions of rabbits following ovulation. These authors suggested a requirement for blastokinin in blastulation. This proposal was challenged by the observation that rabbit blastocysts developed in synthetic media without added blastokinin (Whitten and Biggers, 1968; Kane and Foote, 1970a,b,c). However, a role for blastokinin in subsequent development of the blastocyst is possible.

In the pig, uterine secretion of Fractions IV and V proteins was increased after blastulation, but before blastocyst attachment (Murray

et al., 1972). These data indicated that Fraction IV and V proteins are not necessary for blastulation, but suggested a possible role in subsequent conceptus development.

Hormonal Control of Uterine Secretions

Secretion of total uterine proteins and Fractions IV and V proteins was increased on Days 10 to 16 of the estrous cycle (Murray et al., 1972), and the increase was associated with the period of high progesterone synthesis and release by the pig CL (Hansel and Echternkamp, 1972).

Knight et al. (1973a) examined total protein in uterine flushings of pigs which had been induced to superovulate with pregnant mare serum gonadotropin (PMSG) or which had been unilaterally ovariectomized-hysterectomized resulting in compensatory ovulation on the remaining ovary. Protein concentrations were increased in treated animals as compared with intact control animals. There was a significant correlation between CL weight ($r=.52$) and CL number ($r=.69$) and the quantity of protein recovered. These data suggested that progesterone played an important role in regulating uterine protein secretion. To examine this possibility, Knight et al. (1973b, 1974b) conducted a series of studies. In the first study exogenous progesterone, estradiol or progesterone and estradiol were administered to intact or ovariectomized gilts on Days 4 to 15 after the onset of estrus, and the quantity of protein in uterine flushings was measured (Knight et al., 1973b). Exogenous progesterone increased the concentration of proteins in uterine flushings taken from intact and ovariectomized gilts. Total protein in uterine flushings from gilts given estradiol alone was not different from controls

receiving corn oil; however, administration of progesterone and estradiol increased total uterine luminal protein above values for gilts receiving progesterone alone.

In another study, varied doses of progesterone in combination with varying doses of estradiol were given to gilts and total uterine luminal protein measured (Knight et al., 1974b). Total uterine protein was positively correlated (r=.88) with the dose of progesterone, but negatively correlated (r=-.87) with the dose of estradiol.

Before conclusions are drawn from this work, it is necessary to acquaint the reader with the mechanism of steroid hormone action at target tissues. Complete descriptions have been reported by O'Malley and Means (1974) and O'Malley and Schrader (1976). The initial stage of hormone action at target tissues is hormone binding to a specific cytoplasmic receptor molecule. The hormone-receptor complex is subsequently transported to the nucleus where it binds the chromatin. Hormone-receptor complex interaction with the chromatin leads to the specific response of hormone stimulation. The length of the period of hormone-receptor complex interaction with the chromatin (nuclear retention time) governs the length of the hormonal stimulus.

The interaction of steroid hormone receptors is an important aspect of the uterine response to steroid hormones (see review by Clark et al., 1977). Estrogen binds to its cytoplasmic receptor to form a complex which translocates to the nucleus. This complex is responsible for events leading to increased synthesis of cytoplasmic estrogen and progesterone receptors and increased uterine cellular hypertrophy and hyperplasia. Cells involved in mitosis have a reduced capacity to synthesize and secrete proteins. Progesterone binding to its receptor

initiates events leading to preparation of the uterus for secretory activity. In addition, the progesterone-receptor complex inhibits synthesis of estrogen receptors and decreases nuclear retention time of estrogen receptors thereby limiting the ability of the uterus to respond fully to an estrogen stimulus. Reduction in uterine mitotic activity is of major importance. This steroid hormone receptor interaction stimulates development of a highly secretory uterine endometrium.

The work of Knight et al. (1973a,b; 1974b) suggested that increased uterine endometrial protein secretion is caused by progesterone binding to specific receptors in cells of the uterus. Low concentrations of proteins in uterine secretions from ovariectomized estradiol treated gilts probably reflected a decrease in the number of cells in the synthesis phase of the cell cycle and an increase in the number of cells undergoing mitosis. Progesterone and estradiol receptor interaction was probably responsible for maximal uterine protein secretion in gilts administered progesterone and estradiol.

Role of Uterine Protein Secretions in Fetal Development

According to Bonnet (1882), Aristotle (384-322 BC) and William Harvey (1578-1657 AD) proposed that uterine secretory material supplied nourishment to the conceptus. The effects of uterine secretory proteins on fetal and placental development have been examined by several workers and will be discussed in this review.

In one study, bilaterally ovariectomized and intact pregnant gilts were treated with estradiol and progesterone, and various parameters of placental development were measured on Day 40 of gestation (Knight et al., 1974a). Gilts were assigned to receive either high (3.3 mg/kg) or low

(1.1 mg/kg) doses of progesterone combined with a low (0.55 μg/kg/day) dose of estradiol. Empty uterine weight, allantoic fluid volume and placental length were greater in gilts receiving high doses of progesterone. Conception rate, number of CL, percent embryo survival, number of embryos and dry weight of embryos were not different among treatments. Presumably these effects were mediated, in part, through differences in uterine protein secretion.

In another study (Chen and Bazer, 1973), gilts received intravenous infusions of antiserum to Fraction IV proteins on Days 7, 11, 13, 15, 34, 36, 38, 40 and 42 of pregnancy. Placental length and allantoic fluid protein concentrations were reduced compared with gilts receiving sheep serum; however, fetal dry weight and crown rump length were not affected. The results of these studies suggested that porcine uterine secretory proteins were involved in aspects of placental development which may subsequently regulate development of the fetus. Recently, selected uterine secretory proteins have been isolated, and their physiochemical properties and physiological functions determined.

Uteroferrin

In 1973, Chen et al. described a progesterone-induced basic (pI 9.5-9.7) glycoprotein, purple in color, which was present in high concentrations in uterine flushings from pigs on Days 12 to 16 of the estrous cycle. This glycoprotein, now designated uteroferrin (Uf) (Buhi et al., 1982a) was initially thought to be the only protein present in Fraction IV of uterine flushings as described by Murray et al. (1972). Uteroferrin is synthesized by epithelial cells of uterine glands during the estrous cycle and pregnancy (Chen et al., 1975) with maximal secretion

between Days 60 and 75 of gestation (Basha et al., 1979; Basha et al., 1980). Uteroferrin has a molecular weight of approximately 35,000 by sedimentation equilibrium centrifugation (Buhi et al., 1982b) and contains one molecule of iron (Schlosnagle et al., 1974; Buhi et al., 1982b).

Schlosnagle et al. (1974) reported that Uf possessed acid phosphatase activity; however, the importance of Uf in this capacity within the uterus is questionable considering its pH optimum (pH 4.9) and low specific activity toward available biological substrates.

In 1946, Wislocki and Dempsey observed by histochemical techniques that iron was present in the uterine glands and placental areolae of pregnant pigs. Subsequently, Moustgaard (1969) reported that iron was transported to the fetoplacental unit in protein-bound form. In 1980, Roberts and Bazer suggested that Uf is the major placental iron transport protein in pigs. This proposal was based on the fact that Uf was isolated from allantoic fluid of conceptuses between 30 and 100 days of gestation (Bazer et al., 1975) and that maximum concentrations were recovered on Days 60 to 75 which corresponds to the time of maximum Uf secretion by the uterus (Basha et al., 1979). In 1982, Ducsay et al. determined that Uf was the major iron containing protein secreted by the pregnant uterus. A functional role for Uf in early pregnancy has not been described although it may function to deliver iron to the developing yolk sac placenta which is the first site of hematopoiesis (Patten, 1948).

Retinol Binding Protein

Adams et al. (1981) isolated retinol and retinoic acid binding proteins from uterine secretions of pigs during the luteal phase of the estrous cycle and from allantoic fluid of pregnant animals. The

apparent Kd of the binding protein for retinol (RBP) was 2.6 x 10^{-6}M. Like Uf, RBP was induced in ovariectomized gilts treated with progesterone or progesterone plus estradiol, but not in gilts given estradiol or corn oil (Adams et al., 1981). The RBP comprises a small portion of the total protein in the Sephadex G-200 fraction designated V (Murray et al., 1972). Due to its presence in both uterine secretions of the progesterone stimulated uterus and allantoic fluid, the authors (Adams et al., 1981) suggested that, like Uf, RBP and retinoic acid binding proteins serve to transport water-insoluble nutrients from the maternal uterine endometrium to the conceptus.

Lysozyme and Leucine Aminopeptidase

Lysozyme and leucine aminopeptidase were isolated from the porcine uterus during diestrus and after ovariectomy and administration of progesterone (Roberts et al., 1976). Leucine aminopeptidase activity was associated with protein Fractions I and II, and lysozyme was present in Fraction V after Sephadex G-200 column chromatography (Murray et al., 1972; Roberts et al., 1976). Similar to Uf and RBP, lysozyme and leucine aminopeptidase were detected in allantoic fluid of pregnant gilts which suggested placental transport probably mediated by the placental areolae (Chen et al., 1973; Roberts et al., 1976). A function for these proteins in growth and development of the porcine fetus has not been determined; however, in humans lysozyme is believed to be bactericidal (Sutcliffe, 1975; Galask and Snyder, 1970).

Immune Suppressive Factor

A physiological role was indicated by Murray et al. (1978) for a portion of the acidic proteins of gilt uterine flushings recovered during the luteal phase of the estrous cycle. Uterine flushings from Day 15 suppressed the response of peripheral blood lymphocytes to the mitogen (phytohemagglutinin) and to xenogeneic cells. Uterine flushings from Day 8 were not suppressive except in very high concentrations suggesting that secretion of the mitogenic suppressive activity was controlled by progesterone. Suppressive activity was enhanced in Fraction V after Sephadex G-200 chromatography, indicating that the activity was associated with one of the six progesterone induced acidic proteins described by Squire et al. (1972). The protein(s) responsible for immuno-suppressive activity was not isolated and purified (Murray et al., 1978). Preimplantation mouse embryos express minor transplantation antigens (Palm et al., 1971; Searle et al., 1973; Muggleton-Harris and Johnson, 1976) as is probably the case with other mammalian embryos. Protection from immune rejection by the uterus is necessary; however, the mechanism of this protection is not understood (Beer and Billingham, 1976). It is possible that the mitogenic suppressive activity present in preimplantation uterine secretions from the pig serves to prevent stimulation of maternal lymphocytes, thus allowing implantation to occur. Since secretion of the mitogen suppressive activity was associated with high peripheral progesterone concentrations (Murray et al., 1978), it is likely that secretion continues during pregnancy to protect the conceptuses from immunological rejection.

Protease and Plasminogen Activator Inhibitors

Mullins et al. (1980) reported that uterine flushings taken from nonpregnant gilts on Days 12 or 15, but not 8 or 18 of the estrous cycle inhibited the ability of plasminogen activator to convert plasminogen to plasmin. In addition, plasminogen activator was inhibited by uterine flushings taken from pregnant gilts after Day 12 of gestation or by flushings taken from gilts during pseudopregnancy or after ovariectomy and daily progesterone, but not estradiol, administration (Mullins et al., 1980). These data indicated that plasminogen activator inhibitor (PAI) secretion by the uterus was induced by progesterone. Following Sephacryl S-200 column chromatography of uterine flushings, PAI was associated with a protein peak that had a molecular weight of approximately 15,000.

Recently, Fazleabas et al. (1982) isolated a group of protease inhibitors (PI) in uterine flushings taken from ovariectomized, progesterone treated gilts which inhibited the activity of trypsin, chymotrypsin and plasmin. These protease inhibitors were localized in cells of the uterine glands and surface epithelium by immunocytochemical techniques. In addition, PI appeared to coat and to be taken up by cells of the trophectoderm of the elongating blastocyst.

Proteases secreted by the blastocyst have been implicated in invasive implantation of mammalian trophoblasts into the uterine wall (Denker, 1972). Attachment of the pig conceptus to the uterine endometrium is not invasive but rather involves interdigitation of trophoblast and endometrial epithelia (Amoroso, 1952); however, the pig blastocyst is invasive if placed in an ectopic site (kidney capsule, oviduct or uterine myometrium) (Samuel, 1971; Samuel and Perry, 1972). In addition,

elongated blastocysts secrete plasminogen activator in culture (Mullins et al., 1980), and high levels of plasminogen activator are associated with invasive tumor tissues (Unkeless et al., 1973). Based on these data, Mullins et al. (1980) and Fazleabas et al. (1982) suggested that the role of PAI and PI in uterine flushings is to maintain the noninvasiveness of the porcine blastocyst. In addition, Fazleabas et al. (1982) indicated that PI may prevent destruction of other uterine secretory proteins, required to nourish the conceptus, prior to their uptake by the trophoblast.

The preceding discussion was limited to uterine secretory proteins although other molecules of equal importance are present in uterine secretions. An attempt was made to familiarize the reader with properties and functions of specific protein components of uterine secretions as well as to emphasize the importance of these secretions in fetal development.

Development of the Fetoplacental Unit of the Pig

Development From Fertilization to Day 18 of Gestation

Ovulation of the mature ovum occurs approximately 38 to 45 hours after the onset of estrus in pigs (Oxenreider and Day, 1965; Anderson, 1974) and requires approximately 3.8 hours for completion (du Mesnil du Buisson et al., 1970). Mean ovulation rate estimates range from 12 to 15 ova per animal (Perry and Rowlands, 1962; Linder and Wright, 1978) and the rate is influenced by breed of sow, nutritional status and age (Anderson, 1978). The newly ovulated ovum, invested in granulosa cells reaches the ampullary-isthmic junction of the oviduct in less than 1 hour after ovulation, and the granulosa cells are removed by 1 to 2 hours

after ovulation (Hunter, 1974). Boar sperm are capacitated and reach the site of fertilization within 2 to 3 hours after deposition in the cervix (Hunter, 1974).

Fertilization occurs in the oviduct at the ampullary-isthmic junction, and in pigs the fertilization rate is high with estimates of 90 to 100% (Oxenreider and Day, 1965; Anderson, 1974). Fusion of sperm with the ovum requires that a minimal concentration of divalent cations be present in the oviductal fluid (Yanagimachi, 1978). Apposition of sperm and egg pronuclei occurs 5 to 6 hours after fertilization, and the diploid state is reached by 12 to 14 hours (Hunter, 1974).

The first cleavage occurs 18 to 42 hours after ovulation (2 to 3 days after onset of estrus; Perry and Rowlands, 1962; Hunter, 1974) with the second cleavage 6 to 8 hours later. The second cleavage is staggered with one blastomere dividing earlier than the other as evidenced by embryos in the 3-cell stage (Assheton, 1899; Heuser, 1927). The progeny of the rapidly dividing blastomere continue to divide at a faster rate than the progeny of the other original blastomere. Heuser (1927) suggested that the more rapidly dividing cells yielded the trophoblast cells of the blastocyst, the more slowly dividing cells the inner cell mass. The embryo enters the uterus at the 4-cell stage approximately 30 to 46 hours after ovulation on Day 3 after the onset of estrus, significantly earlier than for other domestic species (Oxenreider and Day, 1965; Hunter, 1974). If the embryo is restricted to the oviduct, development will not proceed beyond the early blastocyst stage (Murray et al., 1971; Pope and Day, 1972). With continued cell division the embryo reaches the morula stage by Day 5 (Heuser and Streeter, 1929; Green and Winters, 1946) and cavitation to form the blastocoel begins soon afterward

(Assheton, 1899; Heuser and Streeter, 1929). Spherical blastocysts with a distinct inner cell mass and trophoblast are present by Day 6 (Assheton, 1899; Heuser and Streeter, 1929; Green and Winters, 1946; Perry and Rowlands, 1962). At this stage the inner cell mass lies beneath the trophoblast.

A period of accelerated growth begins on Day 8 resulting in blastocysts which have a wrinkled appearance that is believed to be caused by an inability of the blastocyst to accumulate fluid at a rate adequate to keep the blastocyst expanded (Heuser and Streeter, 1929). The Day 8 blastocyst is freed from the zona pellucida (Heuser and Streeter, 1929; Green and Winters, 1946; Perry and Rowlands, 1962) by a process believed to involve cycles of blastocyst expansion and collapse and the action of degradative enzymes (Linder and Wright, 1978). Day 8 also marks the beginning of formation of the endoderm. Endodermal cells arise from the inner cell mass and after detachment they proliferate to underlie the trophoblast forming a bilaminar blastocyst (Assheton, 1899; Heuser and Streeter, 1929). During the proliferation of the endoderm, Days 8 to 10, another important process is taking place. The trophoblast cells which overlie the inner cell mass (Rauber's layer) are removed to expose a layer of "embryonic ectoderm" which is disk shaped, continuous with the trophoblast at its edges and bordered beneath by the proliferating endoderm (Heuser and Streeter, 1929; Patten, 1948). Continued expansion of the bilaminar blastocyst occurs during this time. By Day 10 blastocysts expand to a diameter of 5 to 10 mm (Heuser and Streeter, 1929; Dhindsa et al., 1967; Anderson, 1978).

Intrauterine migration of blastocysts to achieve uniform spacing begins on Day 9 but is not completed until after elongation occurs

(Dhindsa et al., 1967). Intrauterine distribution may be accomplished by contractions of the uterine myometrium (Dhindsa et al., 1967) in response to estrogens or other agents of blastocyst origin which are secreted during the initial stages of blastocyst elongation (Perry et al., 1973; Watson and Patek, 1979; Geisert et al., 1982b).

Porcine blastocysts accomplish considerable morphological changes between Days 10 and 12. During this period they undergo successive changes from spherical to ovoid to tubular and then to thin elongated filamentous forms which may obtain a length greater than 1 meter by Day 14 (Heuser and Streeter, 1929; Perry and Rowlands, 1962; Crombie, 1972; Anderson, 1978). During the period of elongation from tubular to elongated forms, blastocysts may expand at a rate of approximately 45 mm/hour (Geisert et al., 1982b). Recently Geisert et al. (1982a) demonstrated that the elongation process involved mostly cellular rearrangement mediated by coordinated movement of endodermal cells lining the cavity of the blastocyst. No increase in the rate of cellular division occurs during elongation.

During elongation the pig blastocyst begins to synthesize estrogens (Flint et al., 1979; Heap et al., 1979; Fischer, 1981). Synthesis and release of this steroid into the uterine lumen is believed to be the signal for maternal recognition of pregnancy in pigs (Bazer and Thatcher, 1977). Recent evidence by Geisert et al. (1982b) indicated that blastocyst estrogen stimulates release of protein secretions, calcium and prostaglandins by the uterine endometrium. In addition, Zavy (1979) reported that total recoverable glucose and fructose in uterine flushings from pigs began to increase between Days 12 and 14 of pregnancy and reached a peak on Day 16. In nonpregnant gilts, the total recoverable

glucose had a similar pattern of change; however, the peak on Day 16 was considerably less than that observed in pregnant gilts (10 mg versus 30 mg). Fructose was not detected in uterine flushings from nonpregnant pigs. Thus early in development, the pig blastocyst acts to insure availability of nutrients to support growth prior to its attachment to the endometrium.

On Days 13 and 14 the epithelial surfaces of blastocyst and endometrium are in close parallel arrangement along the mesometrial border of the uterus (Perry and Rowlands, 1962; Crombie, 1972; Perry, 1981). This is termed the "alignment" phase of attachment by Crombie (1972). Although the blastocyst may obtain a length of 1 meter at this stage, the length of the uterus occupied by each blastocyst is reduced to 25 to 30 cm by intense folding of the uterine endometrial surface and attached trophoblastic membrane (Heuser, 1927; Heuser and Streeter, 1929; Perry, 1981). Blastocysts continue to expand until Day 18 and fill the whole length of the uterus regardless of the number of blastocysts. As a result, longer blastocysts are associated with smaller litters (Perry and Rowlands, 1962; Perry, 1981). Adjacent blastocysts rarely overlap although they touch and may become superficially adhered. Attachment of the trophoblast to the uterus commences on Day 15 (Perry and Rowlands, 1962; Crombie, 1972; Anderson, 1978; Perry, 1981) through discrete areas of cell to cell contact between the epithelium of the trophoblast and uterine mucosa (Crombie, 1972). This arrangement is present on Days 15 to 18 after which it is replaced by microvillar interdigitation of endometrial and trophoblastic epithelial cells over the entire surface of contact (Crombie, 1972; Friess et al., 1980; Perry, 1981).

Immediately prior to elongation of the bilaminar blastocyst, mesodermal cells begin to differentiate from the primitive streak region and extend out between the existing endoderm and ectoderm (Pattern, 1948). After elongation, mesoderm is restricted to the area surrounding the embryo (intraembryonic mesoderm), but by the third week of gestation mesoderm development has spread to encompass the blastocyst and an "extra-embryonic" mesoderm is formed (Patten, 1948; Perry, 1981). The "extra-embryonic" mesoderm plays an important role in formation of the fetal membranes. Soon after start of extraembryonic mesodermal expansion a split appears in the mesoderm forming two layers, one layer closely overlying the endoderm (splanchnopleure), the other underlying the ectoderm (somatopleure; Perry, 1981). The cavity formed by progressive splitting of the mesoderm is the extraembryonic coelom. Development of the embryo proper will not be stressed in the remainder of this discussion to allow a more complete examination of fetal membrane and placental development.

Development of the extraembryonic fetal membranes has been described by many investigators (Heuser, 1927; Heuser and Streeter, 1929; Patten, 1948; Amoroso, 1952; Perry, 1981), and development of these structures (chorion, amnion, yolk sac and allantois) is completed or well underway by the time attachment occurs (Day 18). The chorion is formed by fusion of the outer trophoblastic ectoderm and apposed mesoderm and provides the surface for nutrient, electrolyte and gas exchange with the uterus after attachment. Prior to attachment substances are absorbed directly from the uterine lumen.

The yolk-sac is formed after blastocyst elongation by fusion of splanchnopleuric mesoderm with endoderm (Patten, 1948; Perry, 1981) and

by Days 14 to 15 is present as an elongated tube which runs the length of the blastocyst (Patten, 1948). At this stage in development, the yolk-sac is attached to a small ventral area of the chorion (fused somatopleuric mesoderm and trophoblast; Heuser, 1927) and is surrounded by a vascular plexus which is formed from the overlying mesoderm (Heuser, 1927; Patten, 1948; Perry, 1981). The developed yolk sac is active in transferring nutrients, absorbed from the uterine lumen by the chorion, to the developing embryo (choriovitelline placenta; Heuser, 1927; Patten, 1948; Amoroso, 1952; Perry, 1981). The yolk sac is also active in hematopoiesis reaching a maximum coincidental with the time of its maximal development on Day 18 (Jordan, 1916; Perry, 1981). After Day 18 the yolk sac becomes isolated from the chorion by expansion of the extra-embryonic coelom and rapidly shrinks, becoming relatively inconspicuous by Day 20 (Perry, 1981).

With the demise of the yolk sac, nutrient transport to the embryo is assumed by the developing allantois which after fusion to the chorion becomes the chorioallantoic placenta. The allantois first appears as a caudal extension of the embryonic hindgut on Day 14 (Patten, 1948). This flared extension is composed of an inner layer of endoderm and outer covering of splanchnic mesoderm. With rapid development the distal portion of the allantois grows free of the embryo to form a barrel shaped sac with pointed ends (Day 17). The allantois remains connected with the hindgut through a narrow cylindrical stalk (Patten, 1948). By Day 18, the allantois has an extensive vascular supply formed from the surrounding mesoderm (Patten, 1948) and has expanded to fill a large portion of the extraembryonic coelom. Although not in contact with the chorion at this stage, Heuser and Streeter (1929) suggested that the allantois serves a

nutritive function by absorption and transport of substances which cross the chorion and enter the extraembryonic coelom.

Formation of the amnion begins on Day 14 coincidental with the first appearance of somites in the developing embryo and is accomplished by folding of the chorion over the embryo to create a sac-like enclosure (Heuser and Streeter, 1929; Patten, 1948; Perry, 1981). Fusion of the amniotic folds is completed by Day 18 (Perry, 1981).

Development of the Fetal Membranes From Day 18 to Term

Microvillar attachment of the chorion to the uterus begins on Day 18 (Crombie, 1972; Perry, 1981) and is completed by Day 24 (Amoroso, 1952; Crombie, 1972). At this stage of development the chorion is relatively smooth but folded to accommodate itself to folds of the uterine mucosa (Heuser, 1927). By Day 30, the chorion has become extensively folded to form primary and secondary folds (Heuser, 1927; Crombie, 1972; Friess et al., 1980) which are also termed villi by Heuser (1927); although, true papillary-like villi are not formed. The structure and function of these folds in placental transport will be discussed in conjunction with development of the chorioallantoic placenta.

The allantois expands rapidly and becomes fused to the chorion by Day 19 through their respective mesodermal layers in a region opposite the allantoic stalk (Heuser, 1927; Perry, 1981). By Day 24 the allantois is in contact with the chorion over its entire surface except in the area of the amnion and at the extremities of the chorionic sac (Steven and Morris, 1975). These extremities of the chorion eventually lose their allantoic and chorionic epithelium and degenerate to form the "ischemic ends" of the chorion (Marrable, 1968). Degeneration is attributed to

compromise of the vascular supply to this area of the chorion (Amoroso, 1952). The remainder of the chorion is richly vascularized by the blood vessels of the allantoic mesoderm, and fusion of the chorion and allantois results in formation of a functional chorioallantoic placenta (Steven and Morriss, 1975).

Fluid accumulation within the allantoic sac from Day 18 to 30 is associated with contact and fusion of this membrane to the chorion and with establishment of intimate contact between the chorioallantois and uterine endometrium (Knight et al., 1977). Allantoic fluid volume reaches a peak of 200 to 250 mls on Day 30 but declines to less than 100 mls by Day 45. A second peak measuring between 300 and 350 mls occurs on Day 60 (Knight et al., 1977). Bremer (1916) and more recently Davis (1952) proposed that water formed by action of the mesonephric kidney is the source of water in allantoic fluid. In contrast, Wislocki (1935) studied fetal fluids of the pig and determined that he "could not reconcile the theory of allantoic fluid being in whole or major part an excretory product of the mesonephros" (p. 190) after noting a fetus of less than 3 mm, which lacks a functional mesonephros, with 200 mls of allantoic fluid. In 1957, McCance and Dickerson suggested that allantoic fluid is produced initially by secretion of allantoic membranes and recently Bazer et al. (1981) proposed that allantoic fluid accumulation is the result of fluid transport across the chorioallantois. It was suggested that the estrogen/progesterone ratio is a controlling factor in fluid movement. This was supported by the fact that the estrogen/progesterone ratio was high during accumulation of peak volumes of allantoic fluid on Day 30 and 60 (Knight et al., 1977; Goldstein et al., 1980; Bazer et al., 1981).

Allantoic fluid was originally believed to function solely as a reservoir for fetal urinary waste (Bremer, 1916; Davis, 1952). However, Bazer et al. (1975) reported that allantoic fluid was rich in Uf, a uterine secretory iron transport protein and suggested that the allantois functions as a storage reservoir for substances which cross the chorio-allantois and are later utilized in fetal development. Subsequently carbohydrates and other uterine secretory products were identified in allantoic fluid (Basha et al., 1980; Bazer et al., 1981).

Although the amniotic sac is formed by Day 18 (Perry, 1981), measurable amounts of fluid were not present until approximately Day 30 (Knight et al., 1977; Goldstein et al., 1980). Amniotic fluid volume increased to a peak between Days 70 and 85 and declined thereafter to term (Knight et al., 1977, Goldstein et al., 1980). Changes in amniotic fluid volume and composition do not parallel changes in allantoic fluid which indicates that factors controlling formation of allantoic fluid are of little influence in amniotic fluid formation (McCance and Dickerson, 1957; Knight et al., 1977; Goldstein et al., 1980).

Microvillar attachment of the chorioallantoic membrane to the uterine endometrium marks the beginning of formation of the epithio-chorial placenta of the pig (Grosser, 1909). During formation of the epitheliochorial placenta, fetal chorionic epithelium does not invade the tissues of the endometrium (Grosser, 1909; Amoroso, 1952). According to Grosser (1909), the epitheliochorial placenta is a "strong" barrier to nutrient transport from mother to the fetus because many tissue layers are present between fetal and maternal blood. Although six tissue layers (maternal capillary endothelium, endometrial connective tissue, endometrial epithelium, chorionic epithelium, chorionic subepithelial connective

tissue and fetal capillary endothelium) are present, morphological changes during development of the mature placenta reduce the effective barrier to nutrient transport (Goldstein, 1926; Heuser, 1927; Amoroso, 1952; Dempsey et al., 1955; Amoroso, 1961; Crombie, 1972; Friess et al., 1980).

Soon after attachment of the chorioallantoic membrane to the endometrium, these tissues are thrown into numerous transverse folds or ridges (reviewed by Amoroso, 1952). These primary folds soon subdivide to create the secondary folds which are continuous over the surface of the chorion except at the ischemic ends and at the site of development of the areolae. The chorionic surface is thus divided into areolar and interareolar areas (Abromavich, 1926; Brambell, 1933; Amoroso, 1952).

The chorionic epithelium of the secondary folds has two structural and functional areas: one area made up of the sides and ridges of the folds and another at the base of the folds (fossa; Goldstein, 1926; Heuser, 1927; Crombie, 1972; Friess et al., 1980). By midpregnancy the chorionic epithelial cells of the sides and ridges of the folds are reduced in height compared with the tall columnar cells of the fossa and are deeply indented by basal capillaries. In some cases the chorionic epithelial cell height is reduced to 2 μm by capillary protrusion (Friess et al., 1980). The adjacent uterine epithelium is reduced in height also; however, capillaries remain subepithelial (Friess et al., 1980). In later stages of gestation indentation of fetal vessels into the chorionic epithelium proceeds and the height of the chorionic cytoplasm overlying the fetal capillaries is further reduced (Friess et al., 1980). By late gestation, maternal capillaries are seen to project between the uterine epithelial cells. The modifications of the epithelium markedly reduce the transplacental intervascular distance between maternal and

fetal capillaries which may measure less than 2 μm by term (Crombie, 1972; Friess et al., 1980). Modifications of the placental barrier of the side and ridge of the chorionic folds are indicative of a role for these areas in placental transport of gases and readily diffusible nutrients (hemotroph) and waste (Wislocki and Dempsey, 1946; Friess et al., 1980). Friess et al. (1980) observed pinocytotic vesicles within the apical cytoplasm of the chorionic epithelium and suggested that less diffusible substances secreted by the uterine epithelium are also transported in the interareolar area of the placenta (histotrophic nutrition).

The fossa of the chorionic folds do not undergo extensive reduction during pregnancy nor do fetal capillaries indent the epithelium; however, pinocytotic vesicles suggesting histotrophic nutrient transport are frequently observed at the junction of uterine and chorionic microvilli (Wislocki and Dempsey, 1946; Dempsey et al., 1955; Dantzer et al., 1981). In addition, focal accumulations of uterine secretory material are present in areas where the microvilli are not in close contact, and these probably serve as a source of histotroph for subsequent transfer to the fetus (Wislocki and Dempsey, 1946; Dantzer et al., 1981). The uterine epithelium adjacent to the chorionic fossa remains columnar throughout gestation and capillaries of the rich vascular plexes are subepithelial in location (Dempsey et al., 1955; Crombie, 1972; Friess et al., 1980).

The areolae were first described by John Hunter in 1781 as "white spots" on the placenta of the sow, but their function was not determined until 1828 by Von Baer (Brambell, 1933). Two types of areolae are present on the chorioallantoic membrane, the regular areolae and the irregular areolae (Brambell, 1933). The irregular areolae are present in

small numbers on most chorioallantoic membranes, but are totally absent on others. For this reason their role in placental function is minor and thus will not be discussed further.

Development of the regular areolae begins, on Day 18 of gestation, as an increase in the height of the chorionic epithelium. Development is limited to small circular areas of the chorioallantoic membrane adjacent to the mouths of the uterine glands (Abromavich, 1926; Heuser, 1927; Brambell, 1933). As gestation advances, these areas increase in diameter and invaginate to form a cup-shaped structure with folded walls (Brambell, 1933). The regular areolae are approximately 1 mm in diameter and 1.5 mm in depth (Abromavich, 1926). Due to thickening of chorionic cells around their periphery, the areolae are raised above the chorionic surface and fit tightly into a depression surrounding an opposing uterine gland (Brambell, 1933). Regular areolae appear initially in the central regions of the chorioallantoic membrane and for a time no areolae are present near the ends (Brambell, 1933). However, by Day 50 equal concentrations of areolae are present over the surface of the placenta and number between 2,000 and 2,500 per placenta (Knight et al., 1977). The folded chorionic epithelium within the regular areolae is surrounded by a rich vascular plexus containing fenestrated capillaries (Goldstein, 1926; Brambell, 1933; Friess et al., 1981). Dempsey et al. (1955) reported indentation of capillaries into the epithelium of the areolae, but this observation was not confirmed by Friess et al. (1981).

The primary function of the chorioallantoic areolae is absorption of secretions from the uterine glands to nourish the developing fetus (Abromavich, 1926; Wislocki and Dempsey, 1946; Dempsey et al., 1955; Amoroso, 1961; Friess et al., 1981). Wislocki and Dempsey (1946)

observed histochemical staining for iron in the lumen of the areolae and in vacuoles of the areolar epithelium. These authors proposed that iron containing secretions of the uterine glands are absorbed by the areolae to supply iron for fetal hematopoiesis. Recently, Chen et al. (1975) demonstrated that Uf, an iron containing secretion of pig uterine glands, is absorbed by the areolae. Also, the apical cytoplasm of areolar epithelium contains numerous coated pinocytotic vesicles and tubules which are characteristic of a highly absorptive epithelium (Friess et al., 1981).

In summary, the preceding discussion establishes that fetal nutrient transport proceeds by two routes in the epitheliochorial placenta of the pig. Hemotrophic nutrient transport occurs at the side and on the ridge of the secondary chorionic folds of the chorioallantoic membrane. Histotrophic nutrient transport occurs at the fossa of the chorionic folds by absorption of secretions of the surface epithelium and at the regular areolae by absorption of secretions of the uterine glands.

Maternal Recognition of Pregnancy in Pigs

In pigs, the corpus luteum (CL) is necessary throughout gestation for maintenance of pregnancy (du Mesnil du Buisson and Dauzier, 1957; Heap, 1972). This requirement is presumably due to progesterone, a steroid synthesized and secreted by the CL, which stimulates the development of a highly secretory uterine endometrium to nourish the developing conceptus (Corner and Allen, 1929; Bazer, 1975). In support of this idea, pregnancy was maintained after ovariectomy when progesterone was administered (Day et al., 1959; Spies et al., 1960; Ellicott and Dziuk, 1973).

Corpora lutea of the estrous cycle secrete progesterone in a pattern similar to and at concentrations indistinguishable from the CL of pregnancy until approximately Day 14 after onset of estrus or mating (Guthrie et al., 1972). From Days 14 to 16, progesterone synthesis by the CL of the estrous cycle declines precipitously, while progesterone secretion of the CL of pregnancy is maintained (Guthrie et al., 1972). Functional and morphological regression of the CL and return to estrus (Anderson, 1974) allows another opportunity for successful mating.

Du Mesnil du Buisson (1961) and Dhindsa and Dziuk (1968) examined the influence of the embryo on CL and pregnancy maintenance by the technique of embryo removal. Du Mesnil du Buisson (1961) reported that embryos must be present in both uterine horns between Days 14 and 16 to maintain pregnancy in gilts, and Dhindsa and Dziuk (1968) determined that a pregnancy rate comparable to controls was achieved when embryos were present between Days 12 and 14. A critical role of the embryo in establishment of pregnancy was also demonstrated for the ewe (Moor and Rowson, 1966a; Moor and Rowson, 1966b).

Influence of the conceptus on maintenance of the CL is termed "maternal recognition of pregnancy," and important aspects of control, as they are related to the pig, will be considered in the following discussion.

Hysterectomy of gilts during proestrus and early diestrus resulted in CL maintenance which indicated that the uterus has an important role in determining the lifespan of the CL (Spies et al., 1958; Anderson et al., 1961; Anderson et al., 1969). Anderson et al. (1963) autotransplanted the uterus of prepubertal gilts to the abdominal wall and observed that estrous cycles continued following the pubertal estrus in 4 of 9 gilts

suggesting that uterine control of the lifespan of the CL occurs by release of substances into the blood rather than by neural pathways. Estrous cycles of cows, ewes and pigs were lengthened by intrauterine infusion of substances which destroyed or severely damaged the endometrium (Anderson et al., 1969), which indicates that the uterine luteolysin is produced by the endometrium. This idea was supported by Anderson et al. (1963) who observed estrous cyclicity only in ewes with uterine autotransplants containing histologically normal endometrial tissue. In addition, Schomberg (1967, 1969) reported that uterine flushings taken from gilts on Days 12, 14, 16, 17 and 18 of the estrous cycle were cytolytic when administered to cultured luteal cells. Flushings taken prior to Day 12 and on Day 20 were not cytolytic.

By definition a luteolytic substance causes morphological disruption of luteal tissue which is accompanied by a decline in progesterone production. In 1969 Pharriss and Wyngarden reported that luteolysis occurred in pseudopregnant rats given daily injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Subsequently $PGF_{2\alpha}$ was reported to be luteolytic in the cow (Hafs et al., 1974), ewe (Goding, 1974), mare (Douglas and Ginther, 1975) and pig (Diehl and Day, 1973; Hallford et al., 1974; Kraeling et al., 1975; Moeljono et al., 1976). Prostaglandin $F_{2\alpha}$ is luteolytic in the cow, ewe and mare during the middle and late luteal phase allowing premature CL regression which is necessary to achieve estrus synchronization (Hafs et al., 1974). However, $PGF_{2\alpha}$ did not cause luteolysis before Days 12 to 14 of the estrous cycle in intact pigs (Diehl and Day, 1973; Hallford et al., 1974) and was thus ineffective in reducing estrous cycle length for estrus synchronization (Hallford et al., 1974). Exogenous $PGF_{2\alpha}$ also caused CL regression during pregnancy (Diehl and Day, 1973) and after

induction of pseudopregnancy with estradiol (Kraeling et al., 1975) (pseudopregnancy will be discussed later). These data indicate that $PGF_{2\alpha}$ is luteolytic in pigs; however, it is not effective until after Day 12 following onset of estrus.

Moeljono et al. (1976) administered $PGF_{2\alpha}$ to hysterectomized gilts at various times after onset of estrus to determine if the luteolytic action of $PGF_{2\alpha}$ is uterine mediated. Similar to intact gilts (Hallford et al., 1974), $PGF_{2\alpha}$ caused luteolysis in gilts when injected on Days 14 and 17, but not when injected on Days 8 or 11.

Inability to cause CL regression by $PGF_{2\alpha}$ administration prior to Day 12 is believed to reflect the "autonomous" nature of the CL until this time. Du Mesnil du Buisson and Leglise (1963) reported that CL developed normally in pigs hypophysectomized 3 hours after the onset of estrus. In agreement, Anderson and Melampy (1967) reported that CL of pigs hypophysectomized on the first day of estrus were normal on Day 12 but were regressing on Day 16. These data demonstrate that CL which receive only luteotrophic stimuli associated with the ovulatory surge of luteinizing hormone (LH) are capable of development and function to Day 12. Corpora lutea function in hysterectomized, but not intact gilts was extended to Day 20 by administration of exogenous luteotropins after Day 12 (Anderson et al., 1965; see review Anderson and Melampy, 1967), which indicates that gonadotrophic support alone will not prevent luteal regression when the uterus is present.

Prior to discussion of possible mechanisms regulating the autonomous nature of the early pig CL, it is necessary to describe the mechanism for LH stimulation of progesterone biosynthesis and secretion. Descriptions are based on reports by Henderson and McNatty (1975) and Stryer (1981).

Luteinizing hormone stimulation of luteal cells is mediated by specific receptor molecules in the outer layer of the plasma membrane. The resulting hormone-receptor complex stimulates another membrane protein, the G protein, to exchange bound GDP for GTP. Binding to GTP activates the G protein which stimulates membrane bound adenyl cyclase activity leading to increased intracellular cyclic adenosine mononucleotide phosphate (c-AMP) concentrations. Cyclic-AMP binds the regulatory subunit of protein kinase causing dissociation from the catalytic subunit producing an active protein kinase. Protein kinase is a protein phosphorylator. In the luteal cell it is believed that protein kinase mediates the action of LH by stimulating production of proteins, possibly steroidogenic enzymes, through nucleoprotein phosphorylation or by increasing availability of cholesterol, the biosynthetic precursor of progesterone, through activation by phosphorylation of cholesterol esterase. Activated cholesterol esterase cleaves cholesterol from stored cholesterol esters.

Henderson and McNatty (1975) suggested that autonomy of the porcine CL and refractoriness to $PGF_{2\alpha}$ results from slow dissociation of LH bound at the time of the ovulatory LH surge. After dissociation of a significant number of LH molecules from their binding sites, presumably about Day 12, membrane conformational changes occur. Prostaglandin $F_{2\alpha}$ could then bind possibly to the G protein. Prostaglandin $F_{2\alpha}$ binding would prevent LH activation of adenyl cyclase and lead to CL regression. This mechanism is supported by the work of Caldwell et al. (1969) which indicated that CL induced with human chorionic gonadotropin (HCG) on Days 6, 8, 10 and 16 of the estrous cycle had a functional lifespan of 12.5, 14.5, 15.0 and 19.3 days, respectively, from the day they were induced.

Additional information demonstrating refractoriness of the CL was reported by Krzymowski et al. (1976). Two mg of $PGF_{2\alpha}$ were infused into the anterior uterine vein of gilts for 10 hrs on Days 6, 8, 10, 12, 14 or 15 of the estrous cycle. Prostaglandin $F_{2\alpha}$ had no luteolytic effect on Day 6, 8 or 10 but was effective when given on Days 12, 14 or 15.

Considerable evidence has been reported suggesting that $PGF_{2\alpha}$ is the uterine luteolysin in pigs. Patek and Watson (1976) reported that $PGF_{2\alpha}$ was synthesized and secreted by endometrial tissue taken from the uterus during the luteal phase of the estrous cycle. Previously, Gleeson et al. (1974) reported that increased utero-ovarian vein plasma concentrations of $PGF_{2\alpha}$ were temporally associated with declining peripheral and utero-ovarian vein plasma progesterone concentrations during luteal regression. In a recent study, Moeljono et al. (1977) examined the relationship between utero-ovarian vein plasma $PGF_{2\alpha}$ and peripheral plasma progesterone concentrations from Day 12 to onset of estrus or Day 24 in nonpregnant and pregnant gilts, respectively. Utero-ovarian $PGF_{2\alpha}$ was increased on Days 13 to 17 in nonpregnant gilts and a temporal relationship between CL regression (determined by peripheral progesterone concentrations) and increased $PGF_{2\alpha}$ was observed. In pregnant gilts the number of $PGF_{2\alpha}$ peaks and the average peak heights were reduced compared with nonpregnant gilts, and progesterone concentrations remained elevated. Within each pregnancy status a $PGF_{2\alpha}$ peak concentration was defined as a value greater than two standard deviations above the mean $PGF_{2\alpha}$ concentration for the period studied. These data indicate that endometrial $PGF_{2\alpha}$ is released into the utero-ovarian vein on Days 13 to 17 of the estrous cycle but not pregnancy and is responsible for regression of the corpora lutea in pigs.

Maternal recognition of pregnancy in pigs is probably mediated by signals from the embryo acting on the uterus to prevent transport of the uterine luteolysin ($PGF_{2\alpha}$) to the CL.

In 1977 Bazer and Thatcher proposed that estrogen produced by the blastocyst is the agent responsible for pregnancy recognition in pigs. Previously, Kidder et al. (1955) demonstrated that injection of the synthetic estrogen diethylstilbestrol (DES) on Day 11 of the estrous cycle would prolong the lifespan of the CL. In addition to DES, Gardner et al. (1963) demonstrated that CL were maintained by estradiol or estrone administered on Day 11 of the estrous cycle. Du Mesnil du Buisson (1966) reported that 5 mg of estradiol valerate prolonged CL maintenance when administered daily to nonpregnant, hypophysectomized gilts between Days 12 and 20 and he concluded that estrogen inhibited the luteolytic action of the uterus by blocking secretion or excretion of the luteolysin rather than by modifying a gonadotrophic factor since the pituitary was absent.

Recently, Frank et al. (1977) measured utero-ovarian PGF in gilts injected with 5 mg of estradiol valerate or corn oil between Days 11 and 15 after the onset of estrus. Utero-ovarian vein plasma basal PGF concentrations, PGF peak concentrations and number of PGF peaks were significantly lower in estradiol treated gilts as compared with gilts receiving corn oil. The interestrus interval for gilts given estradiol was 145 days compared with 19 days for control gilts. This extended CL maintenance is termed pseudopregnancy. Patterns of $PGF_{2\alpha}$ release into the utero-ovarian vein were similar to those reported for pregnant versus nonpregnant gilts by Moeljono et al. (1977) and supported the proposal that estradiol of blastocyst origin acts through the uterus to prevent luteolysis (Bazer and Thatcher, 1977).

Perry et al. (1973) first reported that pig blastocyst produce estrogen beginning on Day 12 after the onset of estrus and he suggested that blastocyst estrogen is the luteostatic agent in swine. Subsequently, Fischer (1981) demonstrated that large spherical blastocysts (≈Day 10) produced estradiol-17β. Plasma conjugated estrogens increase steadily from Day 14 to 30 of pregnancy in swine and are believed to be of conceptus origin (Robertson and King, 1974). These data indicate that the conceptus produces estrogen around the time that it is needed to prevent luteolysis. Further support for a role of blastocyst estrogen in maternal recognition of pregnancy in pigs was indicated by the work of Zavy et al. (1980). These investigators reported that estradiol in uterine flushings from pregnant gilts increased between Days 10 and 12 of gestation but that a similar increase was not observed in uterine flushings from nonpregnant gilts. Recently Geisert et al. (1982b) reported that estradiol in uterine flushings from pregnant gilts was associated with the early expansion period of the porcine blastocyst which corresponds to approximately Day 11.5 of gestation.

Failure to observe increased $PGF_{2\alpha}$ in utero-ovarian vein plasma between Days 12 and 20 of pregnancy or pseudopregnancy may be explained by (1) reduced endometrial $PGF_{2\alpha}$ synthesis or (2) continued $PGF_{2\alpha}$ synthesis but reduced release into the uterine venous drainage. To examine this question, Zavy et al. (1980) measured PGF in uterine flushings taken from gilts on Days 6, 8, 10, 12, 14, 15, 16 and 18 of the estrous cycle and pregnancy. Total $PGF_{2\alpha}$ and $PGF_{2\alpha}$ concentrations were higher in pregnant than nonpregnant gilts and a significant Day x Status interaction was detected. In nonpregnant gilts uterine $PGF_{2\alpha}$ concentrations increased from Day 12 to 16, and increasing $PGF_{2\alpha}$ concentrations in

uterine flushings were temporally associated with luteal regression. In pregnant gilts uterine $PGF_{2\alpha}$ concentrations increased steadily between Days 12 and 18 and total recoverable $PGF_{2\alpha}$ was 22.7 μg on Day 18 compared with 0.5 μg on Day 18 for nonpregnant gilts (Zavy et al., 1980). Similar results were obtained by Frank et al. (1978) in nonpregnant gilts given estradiol between Days 11 and 15. Total uterine luminal $PGF_{2\alpha}$ increased from Day 11 to Day 17 and then declined to Day 19 in control gilts, but increased steadily from Day 11 to Day 19 in estradiol treated gilts. These data indicate that low utero-ovarian vein $PGF_{2\alpha}$ concentrations between Day 11 and Day 17 postestrus in pregnant and nonpregnant gilts given estradiol (Moeljono et al., 1977; Frank et al., 1978) probably reflect decreased movement of $PGF_{2\alpha}$ into the uterine venous drainage rather than reduced endometrial $PGF_{2\alpha}$ synthesis.

At this point, it is necessary to discuss the work of Chen et al. (1975) because interpretation of subsequent data is based on their observations. Chen et al. (1975) examined tissue distribution of Uf in the uterus of pigs during the estrous cycle and pregnancy by immunofluorescent techniques. Uteroferrin was localized within the epithelium and lumina of uterine glands on Days 9 and 12 in pregnant and nonpregnant gilts. This distribution is consisted with Uf synthesis and secretion by the uterine glands and is confirmed by recently reported in vitro culture experiments, which indicated that Uf is synthesized by the uterine endometrium taken during diestrus and from Days 10 to 18 and 30 to 105 of pregnancy (Basha et al., 1979; Roberts et al., 1980). In addition, the presence of Uf in lumina of uterine glands (Chen et al., 1975) suggested that Uf was secreted toward the uterine lumen. However, beginning on Day 15 of the estrous cycle, Uf began to be localized within the endometrial

stroma surrounding the glands, and fluorescence within the epithelial cells and lumina of the glands was reduced. Uteroferrin within the uterine stroma, which contains numerous capillaries, would be available for vascular uptake. In pregnant gilts synthesis of Uf by the glandular epithelium and secretion into the glandular and uterine lumen continued to Day 90 of gestation. These data were consistent with the possibility that the direction of Uf release by the endometrium was changed between Days 12 and 15 of the estrous cycle but not pregnancy (Chen et al., 1975). In pregnant gilts this mechanism would allow maintenance and accumulation of histotrophic secretion for conceptus nourishment, while in nonpregnant animals it would facilitate reuptake of secretory macromolecules from the uterus for subsequent utilization and/or degradation. Chen et al. (1975) suggested that a shift in histotroph movement may be mediated by changes in the basement membrane of the glandular epithelium and that local production of steroids by the porcine trophoblast may be involved in its maintenance. Since $PGF_{2\alpha}$ is also a uterine secretory product, these authors suggested that maternal recognition of pregnancy in pigs is mediated by a change in the direction of $PGF_{2\alpha}$ movement in pregnant compared with nonpregnant gilts.

Changes in tissue localization of $PGF_{2\alpha}$ have been reported. Ogra et al. (1974) utilized immunofluorescent techniques to demonstrate that $PGF_{2\alpha}$ is localized within oviductal epithelium prior to ovulation but appears within the lamina propria after ovulation. These authors suggested that observed changes in $PGF_{2\alpha}$ localization may be hormone mediated.

Based on the available data, Bazer and Thatcher (1977) proposed a theory of maternal recognition of pregnancy in swine. During the estrous cycle $PGF_{2\alpha}$ is synthesized and released into the uterine vasculature

(endocrine secretion) and carried to the CL to cause luteolysis. During pregnancy conceptus estrogen alters the direction of movement of $PGF_{2\alpha}$, and secretion is toward the lumen (exocrine secretion) where it is sequestered in large quantities (Zavy et al., 1980). Maintenance of exocrine secretion is important because it prevents $PGF_{2\alpha}$ from reaching the CL to stimulate luteolysis.

The ability of the uterus to sequester or inhibit $PGF_{2\alpha}$ release has been questioned since many physiologists believe that the lipid-like structure of PG affords it the capacity to move freely through cells. This concept of PG movement persists despite considerable evidence to the contrary (Bito, 1972a; Bito, 1972b; Bito, 1974; Bito and Spellane, 1974; Bito et al., 1976). Cell membranes are, in general, impermeable to PGs; however, there are specific carrier-mediated mechanisms across some membranes which facilitate the entry of PGs. In some cells PG movement across membranes requires energy while in others movement is facilitated by counter-transport of other substances (Bito, 1972a). The ability of the uterus to accumulate $PGF_{2\alpha}$ in high concentrations was first reported by Harrison et al. (1972). Uterine fluid obtained from ewes that were rendered acyclic by transplanting the ovary to the neck contained up to 7.6 mg of $PGF_{2\alpha}$. The identity of $PGF_{2\alpha}$ was confirmed by gas chromatography and mass spectroscopy. In subsequent work, a uterine pouch was prepared surgically in ewes, and fluids that accumulated during pregnancy were analyzed (Harrison et al., 1976). Prostaglandin $F_{2\alpha}$ was present in concentrations up to 1500 ng/ml.

Recently, Nkuuhe and Manns (1981) reported that transuterine flux of $PGF_{2\alpha}$ was decreased by infusion of PGE_1 into the uterine lumen and suggested that this mechanism may act to prevent $PGF_{2\alpha}$ release into the

uterine vasculature during pregnancy. However, this effect of PGE_1 has not been confirmed through subsequent studies by these investigators (W. W. Thatcher, personal communication).

The mechanism controlling the direction of PGF movement is not known. Bazer and Thatcher (1977) suggested that integrity of the basement membrane of the uterine epithelium is involved. Basement membranes of glomerular capillaries retard passage of macromolecules with increasing restriction up to 70,000 daltons (<100A) at which point they are effectively prevented from passage (Farquhar, 1978). This upper limit of restriction is greater for basement membranes from vascular endothelium where passage continues for molecules up to 500-700A. These data indicated that a role for the basement membrane in maintaining exocrine secretion of $PGF_{2\alpha}$ was not likely since it has a molecular weight of approximately 300. However, direct measurement of the size limit for basement membranes of epithelial cells is not available (Farquhar, 1978). This fact coupled with the variability in size limits among basement membranes of different tissues indicates that the basement membrane could function to assist in sequestering $PGF_{2\alpha}$. Basement membrane integrity is probably controlled by collagenase since its major structural component is collagen. Recently Woessner (1969) reported that postpartum uterine collagenase activity was inhibited in rats given estradiol. This observation suggests that blastocyst estradiol may act directly on the integrity of the basement membrane.

Tight or occluding junctions (Farquhar and Palade, 1963) function to prevent molecular movement across epithelial layers. They are present between epithelial cells of the uterine surface and glandular epithelium and therefore may function in directing $PGF_{2\alpha}$ movement. Intact tight

junctions are impermeable to small macromolecules and possibly water (see review by Farquhar and Palade, 1963). In addition, degradation of tight junctions between viable cells in tissue culture has been reported (Polak-Charcon and Ben-Shaul, 1979); however, factors regulating this process are not known.

Finally, direction of uterine $PGF_{2\alpha}$ release (exocrine versus endocrine) may be mediated by the secretory mechanism of endometrial epithelial cells which synthesize $PGF_{2\alpha}$. Exocrine release would result from secretion product release at the plasma membrane facing the uterine lumen, and endocrine secretion would result from release at the basal plasma membrane adjacent to endometrial capillaries. This possibility awaits examination.

Other explanations for CL rescue by the embryo consider that estrogen or some protein of the embryo acts directly on the CL rather than endometrium. However, any direct effect of a conceptus product on the CL is questionable given the following: (1) bilateral hysterectomy results in CL maintenance indicating that the luteolysin is of uterine origin and that a conceptus factor is not necessary for CL for maintenance (Anderson et al., 1961); (2) LH infusion into hypophysectomized intact gilts does not rescue the CL, thus gonadotropins do not override the uterine luteolysin (Anderson et al., 1965); (3) unilateral pregnancy does not prevent CL regression suggesting that a systemic conceptus factor is not capable of overriding the uterine luteolysin from the nonpregnant uterine horn (reviewed by Anderson, 1966); (4) antiserum to estradiol 17β or estrone given on Days 10 through 21 of estrous cycle does not prevent CL rescue which indicates that estrogen action is not through a systemic route (Robertson et al., 1980); and (5) the CL of pregnancy is susceptible

to $PGF_{2\alpha}$ induced regression and is not protected by pregnancy (Diehl and Day, 1973). These data strongly argue against a systemic action of conceptus products in CL rescue but are supportive of a local effect of blastocyst secretory products on the uterine endometrium.

This discussion concentrated on data concerned with maternal recognition of pregnancy in pigs. The reader is referred to the review by Bazer et al. (1981) for information on maternal recognition of pregnancy in the cow, ewe and mare.

Placental Iron Transport

The term fetus of placental mammals contains approximately 50 μg of iron per gram of wet tissue (Underwood, 1977), and accumulation of fetal iron in adequate amounts requires efficient transport by the placenta. The major portion of fetal iron is present in erythrocytes and erythropoietic tissues (Pommerenke et al., 1942; Hoskins and Hansard, 1964; Moustgaard, 1969; Ducsay, 1980) as part of the oxygen binding site of hemoglobin. Iron is also a portion of the prosthetic group in the active site of the cytochromes which are enzymes necessary for mammalian cellular respiration (Nicholls, 1974). Oxygen binding to muscle is mediated by myoglobin, a protein which has an iron molecule as part of the oxygen binding site. Myoglobin iron accounts for approximately 10% of total body iron (Åekson et al., 1968). These macromolecules are necessary for fetal growth and development, emphasizing the importance of adequate placental iron transport.

To more fully understand placental iron transport mechanisms, it is necessary to briefly discuss the anatomy and physiology of the placenta.

The mammalian placenta is defined as that area of contact between the fetal membranes and the uterus specialized for the transport of nutrients and gases to the fetus and transport of fetal waste to the maternal system. The placenta is composed of two parts, one of fetal origin continuous with the outer fetal membranes and one of maternal origin (the uterine endometrium). Transport in the placenta occurs, for the most part, by diffusion of nutrients from maternal to fetal blood and is termed "hemotrophic." In other areas of maternal and fetal attachment, nutrient transport occurs by the "histotrophic" route which is characterized by uptake of uterine secretions into the chorionic epithelium.

Grosser (1909) discussed the structure of placentae from several species and proposed a system of classification based on the number of tissue layers present between the maternal and fetal blood vasculature. Originally, four placental types were recognized by Grosser; the epitheliochorial, syndesmochorial, endotheliochorial and hemochorial placentae. Recent data revealed that animals previously believed to possess syndesmochorial placentae do, in fact, have epitheliochorial placentae (Steven, 1975).

The epitheliochorial placenta is considered noninvasive. Chorionic villi interdigitate with folds of the uterine endometrium, but the villi do not penetrate through the uterine surface epithelium. Six tissue layers are present between the blood of mother and fetus. These tissues are maternal capillary endothelium, uterine subepithelial connective tissue (including the epithelial basement membrane), epithelium of the uterine endometrium, chorionic epithelium, fetal subepithelial connective tissue and fetal capillary endothelium. This placental type is found in

the pig, horse and whale (Amoroso, 1952). During the development of the endotheliochorial placenta, fetal chorionic villi invade the uterine endometrium and come to lie in apposition with the maternal capillary endothelium. In this manner the number of tissue layers between the maternal and fetal blood is reduced to four with removal of the endometrial epithelium and subepithelial connective tissue. This class of placenta is present in most carnivores (Amoroso, 1952). The minimum number of tissue layers (three) is found in the hemochorial placenta. Invading fetal chorionic villi form a labyrinth of sinusoids filled with maternal blood, from ruptured endometrial capillaries. Maternal blood is in direct contact with the fetal chorionic epithelium (Steven, 1975). Placentae of rodents and most primates have this tissue arrangement (Amoroso, 1952).

Grosser (1909) suggested that tissues between maternal and fetal blood are a barrier to nutrient and waste transport (via the hemotrophic route) and that the barrier is "strong" in animals with epitheliochorial placentae and "weak" in those possessing hemochorial placentae. Although this system is convenient as a means of classifying placentae based on their morphology, the functional implications with respect to nutrient transport are erroneous. Although placental nutrient, gas and waste transport are influenced by the amount of tissue imposed between maternal and fetal blood, other factors, such as rate and pattern of uterine blood flow, active transport by placental tissues and metabolic rate of placental tissues, are undoubtably important (Steven, 1975).

Pommerenke et al. (1942) was the first to demonstrate transport of maternal plasma iron to the developing fetus. Previously, placental iron transport was believed to proceed solely by phagocytosis of extravasated

maternal erythrocytes into the epithelium of the fetal chorion. At present three mechanisms of iron transport to the fetus are recognized (Burton et al., 1976). In the following review these mechanisms and their relationship to placental type will be discussed.

Absorption of Transferrin Bound Iron From Maternal Plasma

Plasma iron is bound to transferrin during its passage through the mammalian circulatory system. Binding of iron is necessary because plasma transport of iron at concentrations adequate for metabolic requirements would result in formation of ferric hydroxide precipitates (Aisen and Brown, 1977). In addition, transferrin serves as a carrier molecule which can be selectively removed by tissues with high iron metabolism.

Placental transport of iron from maternal plasma is most efficient in mammals with hemochorial placentae (Pommerenke et al., 1942; Nylander, 1953; Bothwell et al., 1958) and proceeds unidirectionally, rapidly and against a concentration gradient (Bothwell et al., 1958; McLaurin and Cotter, 1967). Seal et al. (1972) examined the efficiency of plasma iron transport across placentae of several species, representing all three placental types, by injecting ^{59}Fe into the maternal circulation. Radio-iron content of the fetus was determined at selected times after ^{59}Fe injection, and these values were utilized to determine the percent of the dose transported. In pregnant females with hemochorial placentae, representing eight species, the amount of plasma iron transported by 2 hours was never less than 5% (Rhesus) and was as high as 25% in the chinchilla. Similar results were previously reported for man (Pommerenke et al., 1942), guinea pig (Vosburgh and Flexner, 1950), rat (Nylander,

1953) and rabbit (Bothwell et al., 1958). Bothwell et al. (1958) reported that plasma iron transport in rabbits is adequate to meet requirements of the developing fetus which suggested that this is the only mechanism of iron transport utilized by animals with hemochorial placentae.

The rate of plasma iron transport from mother to fetus is low in animals with endotheliochorial and epitheliochorial placentae (Seal et al., 1972). At three hours after administration of ^{59}Fe to pregnant dogs, sheep, pigs and horses, less than 0.1% of the counts were found in the fetus (Seal et al., 1972). Hoskins and Hansard (1964) report that plasma iron transport to the fetus was still less than 1.0% at 48 hours after administration of ^{59}Fe to pregnant sheep. Similar values were reported for the raccoon by Seal et al. (1972).

Baker and Morgan (1973) and Wong and Morgan (1974) measured plasma iron transport by the endothelial placenta of the cat and determined that only 5 to 10% of the needs of the developing fetus were contributed by this mechanism. Clearly another mechanism of iron transport operates in mammals with endotheliochorial and epitheliochorial placenta.

The first step in plasma iron transport by the hemochorial placenta is binding of maternal transferrin by fetal chorionic villi (Laurell and Morgan, 1964; King, 1976). Laurell and Morgan (1964) reported that transferrin binding was saturable and suggested that binding was receptor mediated. This was supported by immunocytochemical localization of transferrin after binding to the chorion (King, 1976). Transferrin was localized in discrete areas of the plasma membrane of the chorionic epithelium rather than being diffusely distributed over the plasma membrane. Following administration of ^{59}Fe and ^{125}I-transferrin to

pregnant rabbits, Baker and Morgan (1969) observed rapid ^{59}Fe, but slow ^{125}I-transferrin accumulation in the fetus and suggested that transferrin gives up its iron at the placenta for transport to the fetus. Similar results were reported in rats (Graber et al., 1970) and humans (Gitlin et al., 1964). Baker and Morgan (1969) presented data which indicated that iron transport from the chorionic epithelium to the fetal blood is mediated by a small molecular weight molecule. Subsequent work by Larkin et al. (1970) supported this concept. The mechanism of iron removal from transferrin is not known but may involve endocytosis. Endocytosis is believed to be an important step in iron transport from transferrin to reticulocytes (Morgan and Appleton, 1969). Larkin et al. (1970) reported that transferrin was returned to the maternal circulation after iron removal.

Transport of plasma iron by the mechanism described does not appear to involve storage by the placenta prior to distribution to the fetus (Bothwell et al., 1958; McLaurin and Cotter, 1967; Larkin et al., 1970) and increased fetal iron requirements are probably mediated by changes in transferrin binding capacity at the chorion (Baker and Morgan, 1969).

Absorption of Maternal Erythrocytes by Fetal Chorionic Epithelium

Transport of plasma iron was inefficient in animals with endotheliochorial or epitheliochorial placenta (Hoskin and Hansard, 1964; Seal et al., 1972; Baker and Morgan, 1973; Wong and Morgan, 1974). Wislocki and Dempsey (1946) examined the placenta of the cat for iron by histochemical techniques and were unable to demonstrate the presence of iron in the chorionic epithelium. However, an intense reaction was obtained within the chorionic epithelium of the hemophagous region where

there was strong histological evidence of uptake and degradation of maternal erythrocytes. Recently, Wong and Morgan (1974) investigated Fe transport to the fetus by maternal erythrocytes by administering ^{59}Fe labeled plasma or erythrocytes to pregnant cats. They determined that 176 μg of Fe were transferred by maternal erythrocytes and only 2.7 μg Fe were transferred from plasma in 24 hours.

The hemophagous region or hematome is an area of the uterine-fetal membrane interface characterized by the presence of highly phagocytic chorionic epithelium and extravasated maternal blood which is released by erosion of the endometrial capillary endothelium (Amoroso, 1952). This area of blood pooling is differentiated from a true hemochorial placenta by the fact that maternal blood flow in this region is sporadic and proceeds at a low rate (Sinha and Mossman, 1966).

Hemophagous regions have been described in most animals with endotheliochorial placentae (Amoroso, 1952). This fact, together with reported values for plasma Fe transfer (Seal et al., 1972), indicate that phagocytosis of extravasated maternal blood is the major mechanism of Fe transport in these species. This was supported by the report of Wong and Morgan (1974) which demonstrated that phagocytosis of erythrocytes supplied enough Fe to meet the requirements of developing kittens. Wong and Morgan (1974) pointed out the economy of using erythrocytes as an Fe source since each milliliter of blood contains 300 to 500 times the Fe as hemoglobin as is in the form of transferrin iron.

Hemophagous regions were described for the epitheliochorial placenta of sheep (Myagkaya and Vreeling-Sindelárová, 1976) and cows (Bjorkman, 1969) and were located within the placentomes. The quantitative

contribution of the hematome in transport of iron to the fetus in these species is unknown although reported low rates of plasma iron transport (Seal et al., 1972) suggest a major contribution.

Phagocytosis of erythrocytes by chorionic epithelium has been described in detail (Myagkaya et al., 1979; Leiser and Enders, 1980). Briefly, erythrocytes adhere to the apical surface of the chorionic epithelium and are phagocytosed by filopodia-like extensions of the apical plasma membrane. Within the cell, lysosomal enzymes enter the vacuole containing the erythrocyte, and hemoglobin is broken down. In cats accumulations of ferritin granules were associated with degrading erythrocytes, which suggests that freed hemoglobin iron was bound by this molecule (Leiser and Enders, 1980).

Malassine (1977) described ferritin particles, and Leiser and Enders (1980) observed iron deposits in the basement membrane of chorionic epithelium adjacent to the hemophagous region of the cat placenta, indicating that a portion of phagocytized iron was stored. In support of this idea, Wong and Morgan (1974) reported that phagocytosis of maternal erythrocytes delivered twice the iron required by the developing kitten. Hoskins and Hansard (1964) reported evidence that phagocytized iron was stored in sheep. These authors administered ^{59}Fe to pregnant sheep and measured radioiron in fetal and placenta tissues 48 hours after injection. Although accumulation was low, indicative of poor plasma iron transfer, it is likely that a portion of this transport was mediated by phagocytosis of maternal erythrocytes, since adequate time was available for incorporation into maternal hemoglobin. Within the fetoplacental unit most of the iron was present in placental tissues on Day 47 of gestation, suggesting iron storage. With increased demand for Fe as

gestation proceeded the amount of iron present in fetal and placental tissue was similar (Hoskins and Hansard, 1964). These observations suggested that iron was stored in the fetal chorionic epithelium adjacent to the hematome and that increased fetal iron requirements were met by increased release of stored iron rather than by increased uptake of maternal iron.

Absorption of Iron Rich Uterine Secretions

Transport of iron to the fetus by uptake of iron containing secretions of the uterus has been reported in the pig (Wislocki and Dempsey, 1946; Moustgaard, 1969; Chen et al., 1975; Ducsay et al., 1982) and dog (Torbit et al., 1971); however, in dogs this mechanism operates only briefly during development of the placenta and will not be discussed.

Wislocki and Dempsey (1946) were the first investigators to present evidence that iron transport to the developing pig fetus is accomplished by uptake of uterine secretions (histotrophic transport). They observed intense staining for iron in uterine secretions and cells of the chorionic areolae. Only low quantities of iron were observed in cells of the chorionic villi where fetal and maternal vessels would be most closely apposed to accommodate transport of plasma iron. Subsequently, Seal et al. (1972) reported insignificant transport of plasma iron to the pig fetus after injecting ^{59}Fe into pregnant sows near term.

Moustgaard (1969) conducted several experiments to study transport of iron to the pig fetus. In the first experiment radioiron was measured in fetal blood at various time intervals after administration of ^{59}Fe to pregnant sows. Detectable levels of radioactivity were not present until 3 hours after injection, which demonstrated that direct transfer of plasma iron was insignificant.

In a subsequent experiment Moustgaard (1969) administered ^{55}Fe to pregnant gilts to examine the mechanism of histotrophic iron transport. Approximately 8 hours after injection, sections of the intact uterine/placental unit were removed and processed for observation by light microscopic-autoradiography. A high concentration of grains was observed over uterine glands, secretions within the uterine lumen and cells of the chorionic areolae. These observations supported the idea of iron transport by uterine secretions and suggested that the placental areolae play a major role in placental transport of iron.

Chen et al. (1975) examined movement of Uf across the placenta by immunofluorescent techniques and reported intense fluorescence in the areolae which suggested a role for Uf in placental iron transport. To examine this possibility, Ducsay et al. (1982) injected ^{59}Fe into unilaterally pregnant gilts and examined uterine flushings from the nonpregnant uterine horn. Sephacryl S-200 column chromatographic analysis of the flushings revealed that most (67%) of the radioiron counts were associated with Uf. Recently, McDowell et al. (1982) reported that a macromolecule with physiochemical properties similar to porcine Uf was secreted by the endometrium of pregnant mares. Since horses and pigs have similar placental types (epitheliochorial) it is probable that iron transport in horses is mediated by the reported Uf-like molecule.

Iron transported to the fetoplacental unit from uterine secretions appears to be stored prior to uptake by the fetus. Moustgaard (1969) examined radioiron accumulation in pig fetuses and placentae following injection of ^{59}Fe. Large quantities of ^{59}Fe were observed in placentae prior to its appearance in the fetus.

The Fetal Liver as a Hematopoietic Organ

Rapid growth of the developing mammalian embryo leads to increased oxygen requirements for tissue metabolism. Therefore, early development of erythrocytes for transport of oxygen to an increasing tissue mass is necessary. Hematopoiesis is initially localized within the blood islands that form from yolk sac mesenchyme. In the pig, yolk sac hematopoiesis is most active on Day 18 of gestation (Jordan, 1916), but thereafter the liver assumes a primary hematopoietic function. Hematopoiesis in the liver of humans reaches a peak at 5 to 6 months of gestation, but is virtually absent at term (Pearson, 1973). In contrast, liver hematopoiesis continues after birth in pigs and is partially responsible for low postnatal liver iron stores (DuBois, 1963). This review will briefly discuss development of hematopoiesis function in the fetal liver.

To understand fetal liver hematopoietic development, a description of the functional anatomy of the adult liver is necessary. This discussion is derived from the reviews of DuBois (1963) and Junquiera et al. (1977). The main structural component of the liver parenchyma is the hepatocyte. Lacunae run through the liver tissue and in so doing separate the parenchymal cells, creating a system of anastomosing and interconnecting walls (muralium). This muralium is one cell thick in all adult mammals and is bordered on two opposing sides by the lacunae. The lacunae are interconnected with one another by perforations through the parenchymal cell walls creating a sponge-like arrangement of hepatocytes. Specialized capillaries of the liver, the sinusoids, are contained within the lacunae and their walls are formed by an incomplete layer of endothelial and Kupffer cells. This incomplete lining plus endothelial cell

fenestrae allow free movement of macromolecules to the hepatocytes and vice versa. The cytoplasm of the endothelial cell forms a thin layer which lines the sinusoid, and its nucleus is relatively small. Kupffer cells are larger than endothelial cells and contain a large oval nucleus. Their cytoplasm may extend into well defined processes to give them a stellate appearance. Both endothelial and Kupffer cells are phagocytic although the reticuloendothelial derived Kupffer cells are considered the first line of defense. An area termed the space of Disse lies beneath the cells lining the sinusoids and separates these from the hepatocytes. Connective tissue fibers which function to support and maintain the form of the sinusoid lie within the space of Disse.

The muralium of hepatocytes consists of cells arranged in a radial array around a central hepatic vein. This basic unit of liver structure is a liver lobule and forms a polygonal mass of tissue 0.7 mm in diameter and 2 mm long. Branches of the portal vein and hepatic artery are located within connective tissue at the corners of the polygons and form the portal canals. Branches of the hepatic artery and portal vein within the portal canal enter the lobule parenchyma and are continuous with the sinusoids. Blood from these vessels flows through the sinusoids, where molecular exchange occurs and then empties into the central hepatic vein. This lobular structure is most prominent in pigs (Patten, 1948).

Although basic morphological changes in the developing pig liver have been identified (Patten, 1948), a detailed investigation of liver development has not been made. Detailed studies of fetal liver development in mice (Wilson et al., 1963; Rifkind et al., 1969), rats (Bankston and Pino, 1980) and rabbits (Sorenson, 1960; Sorenson, 1963)

are available and these observations are believed to represent the developmental changes common to all mammals. Development of the morphology and hematopoietic function of the fetal liver will be described with reference to observations from the pig, mouse and rabbit.

The fetal liver develops from the hepatic diverticulum which is differentiated as an endodermal thickening in the ventral floor of the foregut on about Day 16 of gestation in the pig (Patten, 1948). In cross-section, the diverticulum appears as a tube of simple columnar epithelial cells surrounded by the mesenchyme of the transverse septum (bodystalk) (Rifkind et al., 1969). A basement membrane surrounds the epithelium. With further development epithelial cords extend radially from the tube to begin formation of the hepatic muralium. Epithelial cells of the cords are not delimited by a basement membrane and come to lie in close contact with surrounding mesenchymal cells with which they may form intercellular junctions (Patten, 1948; Sorenson, 1960; Wilson et al., 1963; Rifkind et al., 1969). Epithelial cords intermingle with both mesenchymal cells and a pair of capillary plexus formed by the two vitelline veins which cross the transverse septum. These vessels develop into the sinusoids of the liver parenchyma and lie in the area between developing hepatic cords (Wilson et al., 1963; Rifkind et al., 1969). Sinusoids of the early liver are completely lined by endothelial cells which are joined at their margins by tight junctions. Macromolecular movement across this lining proceeds through fenestrae within the endothelial cell membrane (Rouiller et al., 1963). Kupffer cells are observed soon after sinusoid development (Rifkind et al., 1969).

Both endodermal epithelium (presumptive hepatocytes) and mesenchymal cells constitute the substance of the cords at this stage. At the

mesenchymal-endodermal interface, the first morphological evidence suggestive of differentiation of endogenous hepatic hematopoietic tissue is discerned (Rifkind et al., 1969). These cells are identified, based on their morphology, as hepatic hematocytoblasts, and their appearance is believed to result from endodermal induced differentiation of the mesenchyme (Rifkind et al., 1969). Hematocytoblasts are undifferentiated hematopoietic cells capable of differentiating into an erythrocyte, granulocyte, megakaryocyte (platelet precursor) or lymphocyte (Junquiera et al., 1977). In the fetal liver, differentiation is primarily in the direction of the erythrocyte.

Definitive commitment to erythropoietic activity is established by differentiation of hematocytoblasts to proerythroblasts, the first stage of erythrocytic differentiation and maturation (Rifkind et al., 1969; Junquiera et al., 1977). Differentiation may be hormonally regulated since sensitivity of hematopoietic stem cells to erythropoietin is established early (Cole and Paul, 1966; Pearson, 1973). Initially proerythroblasts are individually scattered throughout the hepatic cords but after several cycles of mitosis they appear as clusters beneath the sinusoid endothelium. Subsequently the erythron (population of erythrocytes and precursors) out numbers the hepatocytes (Sorenson, 1963). Continued development of erythropoietic tissue involves appearance of progressively more advanced stages of erythrocyte maturity (proerythroblast→basophilic erythroblast→polychromatophilic erythroblast→orthochromatic erythroblast→reticulocyte→mature erythrocyte). All stages of cellular development between hemocytotrophoblast and erythrocyte, including the proerythroblast, will be referred to as erythroblasts in the remaining part of this discussion. Ackerman et al. (1961) reported

that the final stages of extravascular erythropoietic tissue development were achieved by Day 40 of gestation in pigs, and hepatic erythropoiesis continues throughout gestation and for several weeks following birth.

Recently, Bankston and Pino (1980) reported that erythroblasts enter the sinusoids of the fetal liver by migrating through endothelial cells at temporary "migration pores." This process was similar to the transcellular movement of erythrocytes in bone marrow reported by Becker and DeBruyn (1976). Rifkind et al. (1969) observed mostly non-nucleated erythroblasts in liver sinusoids of mice during maximal hepatic hematopoietic activity, but Bankston and Pino (1980) observed a large number of nucleated erythroblasts in sinusoids of rats during the same period. These results suggested that mechanisms which regulate entry of erythroid cells into sinusoids are different among species. Descriptions of the cytology of blood from fetal pigs are not available.

The morphological differentiation of erythroid tissue through the series of stages previously described, involves progressive reduction in cell volume, heterochromatinization, expulsion of the nucleus, gradual disappearance of other cytoplasmic organelles and most importantly accumulation of hemoglobin (see review by Rifkind et al., 1969). The iron requirements for hemoglobin synthesis are extensive. In adult humans, 65% of iron utilization is involved in production of hemoglobin (Dradkin, 1951). This is indicative of the need for efficient mechanisms of iron transport to erythopoietic tissue. In rabbits (Sorenson, 1960; Sorenson, 1963) ferritin bound iron accumulated in the cytoplasm of hepatocytes prior to initiation of erythropoiesis. Ferritin was not present in the hematocytoblast but appeared in the cytoplasm of the newly differentiated proerythroblast, the appearance of which signals the

initiation of erythropoiesis. Sorenson (1963) suggested that hepatic cell iron may accumulate prior to erythropoiesis to supply iron to developing erythropoietic cells; however, Rifkind et al. (1969) did not observe hepatic cell ferritin accumulations prior to initiation of erythropoiesis in the rat.

In adult mammals iron is transported to hepatocytes by transferrin and presumably this mechanism is active during the fetal period (Aisen and Brown, 1977; Aisen and Brown, 1979; Aisen and Listowsky, 1980). Considerable evidence (reviewed by Aisen and Listowsky, 1980) indicates that iron is delivered by transferrin to the erythron although most studies have been performed using only reticulocytes. Delivery of iron by transferrin to hepatocytes (Beamish et al., 1974) and reticulocytes (Aisen and Brown, 1977) is mediated by binding of transferrin to specific plasma membrane receptors. Following transferrin binding to reticulocytes or hepatocytes, iron is released from the protein, at sites and by mechanisms unknown, and ultimately delivered to ferrochelatase for the completion of heme synthesis (Aisen and Listowsky, 1980). Finally, transferrin depleted of its iron, but otherwise intact, is returned to the circulation for another cycle of iron transport.

Other mechanisms of iron transport to the erythron have been described. Policard and Bessis (1958) described ferritin iron transport from a phagocyte to an erythroblast in human bone marrow by a process they termed ropheocytosis. This mechanism proceeded by invagination of a ferritin containing cytoplasmic process into the cytoplasm of an adjacent erythroblast and was completed by formation of a ferritin containing vesicle in the erythroblast cytoplasm. Ropheocytosis of ferritin from endothelial and hepatocytes to erythroblasts in fetal rabbit liver

tissue was reported by Sorenson (1963); however, there was no evidence for this mechanism of iron transport in liver tissue taken from fetal rats (Rifkind et al., 1969).

Systemically injected iron-dextran appeared first in Kupffer and endothelial cells and was later found in hepatocytes (Richter, 1959). These data suggested that iron was transferred to hepatocytes by cells lining the liver sinusoids through a process similar to ropheocytosis; however, there is no direct evidence for this mechanism in adult or fetal liver tissue.

Pinocytosis of serum ferritin also appears to be a mechanism of iron accumulation in erythroblasts. Grasso et al. (1962) and Sorenson (1963) observed ferritin particles in plasma membrane pits and in adjacent cytoplasmic vesicles, which is indicative of endocytotic uptake.

A discussion of development of the hepatic circulation is needed to aid in understanding the relationship of the fetus to placental blood flow and its role in nutrient transport.

Hepatic circulation begins to develop during expansion of the endodermal cords from the hepatic diverticulum. Early in expansion, portions of the vitelline veins are incorporated into the liver as sinusoids. The remaining portions, which are caudal to the liver and drain the gut, anastomose to form the portal vein (Patten, 1948; DuBois, 1963). The umbilical veins are initially embedded in the lateral body walls throughout their course from the belly-stalk to the sinus venosus. With continued growth the liver is forced into apposition with the lateral body walls to which it fuses. The liver sinusoids form connections with the umbilical veins and the blood from the developing placenta is shunted through the liver parenchyma. Meanwhile the umbilical veins distal to

their entrance into the body become fused to form a single vein contained within the umbilical cord. Subsequently, the intraabdominal portions of the umbilical veins lose their paired condition and the right vessel degenerates and no longer carries blood to the liver. The remaining umbilical vein initially empties into the sinusoids of the liver. Eventually a large venous trunk, termed the ductus venosus, forms through the substance of the liver. The ductus venosus carries most of the umbilical blood directly to the inferior vena cava (Patten, 1948; DuBois, 1963). However, a significant portion of the umbilical circulation empties into the liver sinusoids through branches leaving the ductus venosus. Thus, the liver is able to metabolize nutrients immediately after their transport to the blood from the placenta. The described vascular arrangement is completed between Days 25 and 30 in the pig and persists until parturition (Patten, 1948).

CHAPTER III
CELLULAR ASPECTS OF EXOCRINE-ENDOCRINE MOVEMENT OF UTERINE SECRETIONS

Introduction

Progesterone synthesized by ovarian corpora lutea (CL) is needed for maintenance of pregnancy in pigs (du Mesnil du Buisson and Dauzier, 1957; Ellicott and Dziuk, 1973). Peripheral plasma progesterone concentrations in pregnant and nonpregnant gilts are similar to Day 14 after the onset of estrus (Guthrie et al., 1972); however, between Days 14 and 16 of the estrous cycle peripheral progesterone concentrations decline rapidly to low concentrations (<1 ng/ml) prior to onset of estrus. Progesterone concentrations remain elevated (10-20 ng/ml) in pregnant gilts (Guthrie et al., 1972). Reduction of peripheral progesterone concentrations in nonpregnant gilts is associated with regression of the CL (Caldwell et al., 1969), and the uterus plays an important role in regulating life-span of the CL (Anderson et al., 1961). Corpus luteum regression can be induced in pigs by administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Hallford et al., 1974), and its production by the uterine endometrium of cyclic and pregnant gilts has been reported (Patek and Watson, 1976). Moeljono et al. (1977) reported that $PGF_{2\alpha}$ concentrations in blood from the utero-ovarian vein were increased on Days 13 to 17 of the estrous cycle, but not pregnancy, and this increase was associated with CL regression. These data suggested that $PGF_{2\alpha}$ is the uterine luteolytic factor in pigs and that the presence of conceptuses in the uterine lumen prevents release of

$PGF_{2\alpha}$ into the utero-ovarian vein, thereby allowing maintenance of CL function (luteostasis). Dhindsa and Dziuk (1968) demonstrated that embryos must be present in the uterine lumen between Days 12 and 14 of pregnancy to maintain subsequent CL function. Corpora lutea were maintained (Gardner et al., 1963), and utero-ovarian vein $PGF_{2\alpha}$ concentrations on Days 13 to 17 after onset of estrus reduced (Frank et al., 1977) following administration of 5 mg estradiol to nonpregnant gilts between Days 11 and 15 of the cycle. Perry et al. (1976) demonstrated that pig blastocysts begin to produce estradiol on about Day 10 or 12 of pregnancy, and these investigators proposed that the blastocyst signals its presence by release of this steroid hormone. Recognition of this signal would lead to reduced release of $PGF_{2\alpha}$ into the utero-ovarian vein and maintenance of uterine secretory activity to nourish the fetus. These maternal adjustments in response to the presence of the embryo constitute maternal recognition of pregnancy. Recently, Bazer and Thatcher (1977) proposed a theory of maternal recognition of pregnancy in swine to explain the action of estradiol on uterine release of $PGF_{2\alpha}$. These investigators proposed that estrogen produced by the blastocyst acts locally on the uterine endometrium to influence the direction of movement of $PGF_{2\alpha}$ after its synthesis. This concept was based on a study by Chen et al. (1975) which investigated, by immunofluoresence, the uterine endometrial tissue distribution of uteroferrin (Uf), a uterine secretory product. In nonpregnant gilts, Uf was present in cells and lumina of endometrial glands through Day 12 of the estrous cycle (Chen et al., 1975). This distribution pattern was indicative of Uf movement toward the uterine lumen (exocrine secretion). By Day 15 fluorescence was reduced in cells and lumina of the glands but had become intense in stroma surrounding the

glands. These observations suggested that Uf was passed to the endometrial stroma where it could enter the systemic circulation (endocrine secretion). In pregnant gilts, exocrine release of Uf continued (Chen et al., 1975). Bazer and Thatcher (1977) proposed that uterine secretory products, specifically $PGF_{2\alpha}$ and Uf, are secreted in an exocrine fashion until Day 12 in nonpregnant gilts after which secretion becomes endocrine in direction. Estrogen produced by the blastocyst would mediate continued exocrine secretion in pregnant gilts, which would prevent $PGF_{2\alpha}$ from reaching the CL and causing luteolysis and facilitate continued secretion and accumulation of uterine secretions for fetal nourishment. This concept was supported by the work of Zavy et al. (1980) and Frank et al. (1978) which demonstrated that Uf and $PGF_{2\alpha}$ were sequestered in the uterine lumen during early pregnancy and after induction of pseudopregnancy by estrogen administration, respectively. In nonpregnant gilts, uterine luminal total Uf and $PGF_{2\alpha}$ increased to Day 12 but declined thereafter (Frank et al., 1977; Zavy et al., 1980). Bazer and Thatcher (1977) suggested that changes in basement membrane permeability may be responsible for the shift in direction of movement of uterine secretions. This idea was based on the fact that (1) the primary structural component of basement membrane is collagen, and (2) uterine collagenase activity is reduced after estrogen administration to rats (Woessner, 1969). However, direct evidence for a role of this or any other cellular mechanism in regulating endocrine-exocrine release of uterine secretory products is not available.

The primary objective of this study was to investigate cellular mechanisms responsible for the shift from exocrine to endocrine release of uterine secretory products. The uterine glandular epithelium was

examined by various methods with emphasis directed to (1) the integrity of the basement membrane for the reasons described, (2) the occluding junctions located between adjacent epithelial cells since these structures are believed to be impermeable to small molecules (see review by Farquhar and Palade, 1963) and (3) the direction of release of secretory products synthesized by the endometrial glands. Observations were also made in relation to the cellular aspects of protein secretion with special emphasis on the synthesis and release of Uf.

Materials and Methods

Animals and Surgical Procedures

Gilts with two previous estrous cycles of normal duration (18-22 days) were checked daily for estrus with intact boars and bred when estrus was detected and at 12 and 24 hours after detection of estrus. Nonpregnant gilts were managed similarly except that boars were not allowed to breed. Surgical procedures were performed with gilts under general anesthesia, and tissues were obtained after exposure of the reproductive tract by midventral laparotomy (Knight et al., 1977).

Endometrial samples taken from gilts on Days 8, 10, 12, 15 and 18 of gestation and the estrous cycle (n=3, 3, 4, 3 and 3 for nonpregnant gilts; n=3, 3, 3, 3 and 3 for pregnant gilts) were used to determine collagenase-like enzyme activity, total and free hydroxyproline and percent dry weight. Uterine gland ultrastructure and acid phosphatase cytochemistry were examined in nonpregnant and pregnant gilts on Days 10, 12, 15 and 18 after onset of estrus (n=2, 4, 3 and 1 for nonpregnant

gilts; n=1, 3, 3 and 3 for pregnant gilts). Immunoperoxidase was used to localize Uf in tissues taken from nonpregnant and pregnant gilts on Days 8, 10, 12, 15 and 18 after onset of estrus (n=10).

Preparation of Tissue Extracts for Assay of Collagenase-Like Activity

Collagenase-like activity (CLA) was determined in endometrial tissue extracts prepared as described by Weeks et al. (1976) for extraction of the postpartum rat uterus. Endometrial tissues were taken from the uterus after hysterectomy and rinsed in ice cold saline. Endometrium (∿15 gms) was minced and homogenized with several short bursts of a polytron (Brinkmann Instruments, Westbury, NY), using 10 ml of homogenization fluid (0.01 M $CaCl_2$, 0.25% (v/v) Triton X-100) per gram of wet tissue. The homogenate was centrifuged at 6000 x g for 30 min and the supernatant, designated Fraction I, was poured off and saved. The pellets were recovered and resuspended briefly in a volume of resuspension buffer (0.05 M Tris, 0.1 M $CaCl_2$, pH 7.4) equal to that used in the original homogenization. The homogenate was distributed to stainless steel centrifuge tubes and incubated in a water bath at 60°C for 4 minutes. After incubation, tubes containing the homogenate were cooled by immersion in an ice bath and then centrifuged at 7500 x g for 30 minutes. The supernatant, designated Fraction II, was poured off and saved. Samples (Fractions I and II) were dialyzed in the cold (4°C) overnight against 1 mM Tris-HCl (pH 7.5) with at least one change of the dialysate. Aliquots (100 ml) of each fraction were lyophilized and the resultant material resuspended in 5 ml of assay buffer (0.04 M Tris-HCl, 0.01 M $CaCl_2$, 0.15 M NaCl, pH 7.5). Samples were assayed for CLA immediately after resuspension.

Preparation of Rat Tail Collagen

Purified rat tail collagen was prepared by a modification of the method of Strawich and Nimni (1971) which described the isolation of collagen from bovine articular cartilage. Briefly, tendons were removed from excised rat tails, minced with scissors and dissolved in 0.5 M acetic acid (1 ml/mg tendon). Dissolved tendons were centrifuged at 10,000 x g for 30 mins and the supernatant retained. Sodium chloride was added to the collagen solution to a concentration of 5% (w/v) with slow stirring. Following addition of NaCl the solution was stirred for an additional 15 min and centrifuged at 10,000 x g for 30 mins. The pellet was resuspended in cold 0.5 M acetic acid and addition of 5% (w/v) NaCl and centrifugation were repeated. The resultant pellet was resuspended in cold 0.45 M NaCl adjusted to pH 7.0, and this solution was salted out with 20% (w/v) NaCl and centrifuged at 10,000 x g for 30 mins. The pellet was resuspended (0.45 M NaCl) and dialyzed (0.45 M NaCl) overnight. Following centrifugation at 100,000 x g for 90 min, the resultant supernatant was dialyzed (0.15 M sodium phosphate buffer, pH 7.4) and stored at 4°C as a source of purified collagen. All procedures were performed at 4°C.

Strawich and Nimni (1971) reported that the purified collagen obtained by this procedure resulted in a single component, following denaturation, with the same electrophoretic mobility, elution pattern from carboxymethylcellulose and sedimentation velocity as the $\alpha 1$ chain from skin collagen. However, in the present study, purity of the collagen component obtained was not determined.

Iodination of Purified Collagen

Purified collagen was iodinated by the Iodo-Gen technique (Markwell, 1978). Acid washed 13 x 100 mm tubes were coated with 100 µg of Iodo-Gen (1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycoluril) (Pierce Chemical Company, Rockford, IL) by evaporation of chloroform. Collagen (1 mg) in 1 ml of iodination buffer (0.075 M sodium barbital, 0.4 M NaCl, pH 8.0) was added to the Iodo-Gen tube previously rinsed in buffer. Carrier-free Na ^{125}I (0.5 mCi) was added and the tube shaken a few seconds every minute for a total of 10 min. The ^{125}I-collagen was separated from unreacted Na ^{125}I by chromatography at 4°C on a Sephadex G-25 disposable column equilibrated with iodination buffer. Specific activity of the iodinated sample was approximately 2.9 x 10^8 cpm/mg. Each molecule of collagen contains 4 tyrosine residues (≈2500 total amino acids) available for iodination, and iodination of native collagen by oxidation methods (Chloramine T) to sufficient specific activity for use in radioimmunoassay has been previously demonstrated (Adelmann et al., 1973).

Assay of Collagenase-Like Activity

Collagenase-like activity in endometrial extracts was determined by a modification of the procedure of Johnson-Wint (1980). Collagen plates were prepared by addition of 0.025 ml (5000 cpm) iodinated collagen (2 mg/ml; 200,000 cpm/ml) to each well of a 96 well micro-titer plate (Falcon, Oxnard, CA). Plates were allowed to gel by incubation for 1 h at 37°C. Collagenase-like activity was determined by incubating tissue extracts (0.2 ml) on ^{125}I-collagen gels for 3 h at 37°C and measuring the quantity of ^{125}I-products released. Control incubations were run in the presence of assay buffer alone. One relative unit (RU) of activity was

defined as the ability to release 5% of the ^{125}I solubilized by 0.025 ml of a commercial preparation of collagenase (4 mg/ml; Type III collagenase; Sigma, St. Louis, MO). Fractions I and II were assayed individually; however, the reported results represent the sum of these values.

Preliminary experiments indicated that the enzymatic activity in endometrial extracts was only partially inhibited ($\sim$45%) by addition of ethylenediamine tetraacetic acid (EDTA; 10 mM) to bind free calcium. Addition of a serine protease inhibitor (phenylmethylsulfonylfluoride; 1.0 mM) to extracts also reduced solubilized counts ($\sim$45%), but the inhibitory effect of phenylmethylsulfonylfluoride (PMSF) and EDTA were not additive when used in combination (52%). These data suggested that inhibition of collagen digestion by these substances was occurring by a common mechanism, probably inhibition of a trypin-like protease, since trypsin is inhibited by PMSF binding at the active site and by removal of calcium from assay media. Soybean trypsin inhibitor (200 μg; Sigma, St. Louis, MO), which also inhibited digestion of collagen by approximately 50%, was added to all samples plus total count and buffer control tubes to eliminate digestion caused by this protease. Additional soybean trypsin inhibitor was not added to portions of samples previously treated with trypsin and excess soybean trypsin inhibitor to activate CLA. The enzymatic activity toward collagen not associated with serine proteases was defined as "collagenase-like activity", since this activity was, for the most part, not calcium activated. All mammalian collagenases which have been isolated require calcium for their activity (reviewed by Gross, 1976). This suggests that the enzymatic activity measured by the described procedure is not that of a classical collagenase; however, its ability to digest purified fibrillar collagen indicates that it may be of physiological importance.

Collagenase-like activity determined in untreated aliquots of endometrial extracts was defined as "active" CLA. This portion of measured activity is believed to be that available for activity without further modification of the enzyme. Woessner (1979) previously identified this portion of collagenase activity in uterine extracts from postpartum rats. Another portion of collagenase enzyme, termed latent collagenase has been reported in uterine tissue from rats and activation of this enzyme requires addition of serine proteases (Woessner, 1979). Latent collagenase may be considered reserve enzyme which is activated when needed. Total collagenase is the sum of active and latent collagenase activity and may be determined by pretreating collagenase solutions with trypsin prior to determination of enzymatic activity (see next section).

Activation of Collagenase-Like Activity

Aliquots (1 ml) of endometrial Fractions I and II were incubated with 50 μg of trypsin (Sigma, St. Louis, MO) for 5 min at 37°C. After incubation 200 μg soybean trypsin inhibitor was added and the mixture incubated for 5 min at 37°C (Kitamura et al., 1980).

Protein Measurement

The quantity of protein in endometrial extracts was determined by the method of Lowry et al. (1951) using bovine serum albumen (BSA) as standard.

Determination of Endometrial Total Hydroxyproline

Immediately after hysterectomy, endometrial samples (triplicate) were weighed (∿0.5 gm) and prepared for total hydroxyproline determination

by an adaptation of the method described by Woessner (1961). Tissues were minced and transferred quantitatively to Pyrex test tubes with screw caps for hydrolysis. Samples were hydrolyzed in 5 ml of HCl (6 N) for 3 h at 135°C. After hydrolysis, the sample and distilled water rinsings of each tube were filtered (Whatman #2; Whatman Ltd., England) into 25 ml volumetric flasks. Several drops of 0.02% (w/v) methyl red indicator were added and NaOH (2.5N) was added to neutrality. Samples were diluted to 25 ml and frozen (-20°C) until assayed for hydroxyproline.

Hydroxyproline was determined in aliquots (0.025 µl) of prepared samples by "Method I" as described by Woessner (1961). This procedure involved oxidation of hydroxyproline, within each sample solution, by reaction with Chloramine T. Oxidized hydroxyproline was reacted with p-dimethylaminobenzaldehyde (pDMAB), and quantification of the proline-pDMAB compound was achieved by measurement of absorbancy of the solutions at 557 nM. All samples were assayed in duplicate.

Determination of Endometrial Free Hydroxyproline

Triplicate samples of endometrial tissue were weighed (∿0.5 g) and prepared for determination of free hydroxyproline by the method of Woessner (1969). Hydroxyproline was determined in duplicate by "Method II" as described by Woessner (1961). This method was similar to "Method I" previously described for determination of total hydroxyproline; however, unreacted pDMAB which may interfere with accuracy of absorbancy deterinations was removed by extraction with benzene. This was necessary due to the low levels of hydroxyproline in samples prepared for determination of free hydroxyproline.

Determination of Endometrial Dry Weight

Endometrial samples (triplicate) were weighed in tared aluminum pans and dried to constant weight in an oven at 80°C.

Immunocytochemical Localization of Uteroferrin

Tissues were cut to 3-5 mm^3 pieces and fixed overnight at 4°C by immersion in a solution of freshly prepared 2% (w/v) paraformaldehyde, 0.1% (w/v) glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.0. Fixed tissues were washed three times (30 min each wash) in buffer at 25°C, dehydrated in a graded series of ethanols (70 to 100%) and embedded in paraplast (Lancer Mfg., St. Louis, MO). Sections (7 μm) were cut, floated onto a solution of water plus gelatin (0.075%) at 45°C and then mounted on glass slides.

Uteroferrin was localized in tissue sections by the peroxidase-antiperoxidase (PAP) bridge technique (Moriarty et al., 1973) utilizing the double bridge modification reported by Ordronneau (1979) which increases staining of localized tissue antigens. Antiserum to Uf was produced by the method of Chen et al. (1973) and used at a dilution of 1:10,000 in phosphate buffered saline containing 1% (v/v) normal rabbit serum (PBS/NRS). Freshly deparaffinized tissue sections were rinsed in PBS for 3 min and subsequently incubated with 2% (v/v) PBS/NRS for 3 min to reduce nonspecific staining. Treatment with normal rabbit serum was repeated prior to adding each PAP component. Solutions were added dropwise to horizontally placed slides. After the first PBS/NRS rinse, sections were incubated with antiserum to Uf for 48 h at 4°C in a humidified chamber. To terminate the incubation, slides were washed three times by immersion in 200 ml of PBS (3 min each wash). The second PAP

component, rabbit antisheep gamma globulin (Cappel Laboratories, Cochranville, PA), was diluted to 1:100 with PBS/NRS, incubated with the tissue for 10 min at room temperature and washed as before. The third PAP component, sheep peroxidase-antiperoxidase (Cappel Laboratories, Cochranville, PA), was added to sections at a dilution of 1:150 for 10 min at room temperature. The incubation was terminated by washing with PBS. To achieve formation of the double bridge (Ordronneau, 1979), rabbit antisheep gamma globulin and sheep peroxidase-antiperoxidase were reapplied stepwise to the tissue as previously described. To stain the tissue, a solution of 75 mg/100 ml of 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) in 0.05 M, pH 7.6 Tris-HCl buffer was prepared (Nakane and Pierce, 1966), filtered (Whatman #1; Whatman ltd., England) and hydrogen peroxide added to a final concentration of 0.002% (w/v; Petrusz et al., 1975). Slides were immersed in this solution for 8 min with constant slow stirring. The brown precipitate formed by peroxidase oxidation of DAB allowed visual localization of the antigen-antibody-enzyme complex. The slides were then washed with 0.05 M Tris-HCl buffer (pH 7.6) for 2 min and finally in PBS for 2 min. Some sections were counterstained with Mayer's hematoxylin for 4 min. The control experiments described below were conducted to determine the specificity of the PAP procedure (Petrusz et al., 1976). In the first experiment, components of the stain were sequentially replaced by PBS to ascertain the staining contribution of each component through direct binding to the tissue. The second experiment involved application of the staining procedure to porcine spleen, a tissue known to be devoid of Uf (Chen et al., 1973), to detect non-specific staining. In the third control experiment, the primary antiserum was used at sequentially higher dilutions. With this

procedure, those antibodies within the serum which bind tissue antigens, but are present in lower quantity than the antibody of interest are reduced below detectable limits. Thus, staining of areas by undetermined antibodies can be identified. The fourth control involved absorption of the specific antiserum with increasing amounts of purified Uf for 2 days at 4°C before PAP staining. This procedure is the only direct method to establish the specificity of the reaction to the tissue protein. As a final control, NSS was used instead of antibody to Uf. In addition to tissue control experiments, the primary antiserum was utilized in a radioimmunoassay (see Chapter IV, Placental Transport and Distribution of Uteroferrin in the Fetal Pig) and did not crossreact with either transferrin or lactoferrin, two iron containing glycoproteins with some similarities to Uf (Roberts and Bazer, 1980) and found in biological fluids. Tissue sections were examined for staining and photographed using a Wild M20 compound microscope.

Electron Microscopy: Ultrastructure

Endometrial tissues were cut to 1 mm^3 pieces and fixed for 2 h at room temperature by immersion in a solution of 2% (w/v) glutaraldehyde in cacodylate-HCl buffer (0.1 M, pH 7.2). Fixed tissues were washed three times (10 min each) with cacodylate-HCl buffer (0.1 M, pH 7.2) and then postfixed in 1% (w/v) osmium tetroxide for 1 h. Washing was repeated, and tissues were dehydrated through a graded series of ethanols (25 to 100%), transferred to acetone and embedded in Spurr's low viscosity embedding medium (Spurr, 1969). Thin sections (80-90 nm) were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome and collected on formvar coated copper grids. Sections were stained with saturated uranyl

acetate for 10 min followed by lead citrate for 10 min (Reynolds, 1963). Sections were examined and photographed on a JEOL 100-CX transmission electron microscope. A minimum of three glands per block and five blocks per animal were examined.

Electron Microscopy: Acid Phosphatase Cytochemistry

Endometrial tissues (1 mm^3 pieces) were fixed for 2 hr at room temperature by immersion in a solution of 2% (w/v) glutaraldehyde in cacodylate-HCl buffer (0.1 M, pH 7.2). Fixed tissue was washed twice (30 min each) at room temperature and held overnight at 4°C in cacodylate buffer (0.1 M, pH 7.2).

Acid phosphatase activity was localized by a modification of the technique described by Miyayama et al. (1975). All buffers contained distilled water that had been boiled to reduced the content of carbon dioxide. Tissue sections (~150 μm) were cut with a hand microtome (Lewis and Knight, 1977) and transferred to cold sodium acetate buffer (0.1 M, pH 4.9). For acid phosphatase localization, tissue sections were incubated 1 h at 37°C in a solution of p-nitrophenyl phosphate (3.0 mM) and lead nitrate (3.6 mM) in sodium acetate buffer (0.1 M, pH 5.0). Control incubations were run without the addition of the substrate, p-nitrophenyl phosphate. Following incubation, sections were washed three times (15 min each) in cold cacodylate buffer (0.1 M, pH 7.2). Postfixation, degradation, embedding and thin sectioning were performed as described in the previous section (Electron Microscopy: Ultrastructure). Sections were stained with saturated uranyl acetate for 10 min

at 45°C followed by lead citrate (Reynolds, 1963) for 30 min at room temperature. Sections were examined and photographed on a JEOL 100-CX transmission electron microscope.

Basement Membrane Thickness

Endometrial tissues previously prepared for acid phosphatase cytochemistry were used to obtain basement membrane data. The basal portion of one cell in each of three glands from each animal was photographed at 50,000x on a JEOL 100-CX transmission electron microscope. The basement membrane was measured on the developed negative at two places and each measurement represented a single observation. Basement membrane measurements were taken in an area adjacent to a portion of the plasma membrane with easily identifiable trilaminar structure, indicative of a near perpendicular plane of sectioning.

Statistical Analysis

Data were analyzed by least squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (Barr et al., 1979). The overall mathematical model for all variables (collagenase-like activity, total hydroxyproline, free hydroxyproline, percent dry weight and basement membrane width) included effects of status (pregnant versus nonpregnant), day and status by day interaction. Data were separated by status and analyzed individually for day trends.

Results

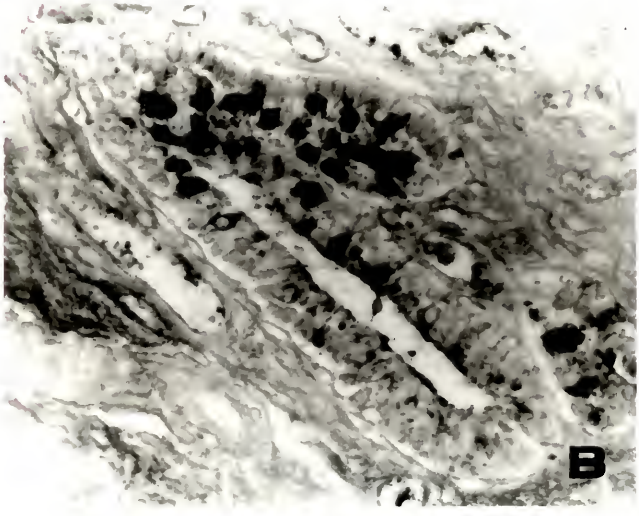
Immunoperoxidase Localization of Uteroferrin

Localization of Uf was similar in pregnant and nonpregnant gilts on Days 8, 10, 12 and 15 after onset of estrus. Positive staining for Uf was not observed on Day 8 (Fig. 3.1a). On Day 10, Uf was observed in epithelial cells, but not in lumina, of uterine glands which suggested that Uf was being synthesized but not released (Fig. 3.1b,c). In addition, staining for Uf was not observed for all glands within a section of tissue, and Uf was not present in all cells of a gland containing staining. The pattern of Uf staining in cells of the uterine glands on Day 12 was similar to that observed on Day 10. In addition, staining was associated with fibrillar material within the lumina of the glands, which was indicative of Uf secretion (Fig. 3.1d). On Day 15, Uf was observed in epithelial cells and lumina of all glands examined, and most cells within each gland were positively stained (Fig. 3.1e). Staining was located throughout the cytoplasm, although some localization of staining was apparent within the apical portion. The pattern of Uf localization on Day 18 of pregnancy was similar to that observed on Day 15 of the estrous cycle and pregnancy. However, on Day 18 of the estrous cycle positive staining for Uf was markedly reduced (Fig. 3.1g). Staining was restricted to a small supranuclear area in epithelial cells of the uterine glands. In addition, large cells with clear cytoplasm were present within the glandular epithelium. These cells did not contain Uf (Fig. 3.1h).

Uteroferrin was not localized within cells of the luminal epithelium of the uterus or in the endometrial stroma in any of the tissues examined. Positive staining was associated with the apical surface of the luminal

Figure 3.1. Immunoperoxidase localization of Uf in uterine glandular epithelium of pregnant and nonpregnant gilts. X1280 for (A)-(G). A) Uterine glands from Day 8 of gestation. Uteroferrin was not detected. B) Positive staining for Uf in a uterine gland on Day 10 of the estrous cycle. Staining is supranuclear in the cells that contain Uf. C) Adjacent section to that shown in B but with NSS used in place of antiserum to Uf. D) Uterine glands from Day 12 of the estrous cycle. Note presence of Uf staining material in the lumen of the glands (arrows). E) Diffuse positive staining of a uterine gland from Day 15 of gestation. Supranuclear concentration of staining is still present (arrows). F) Uterine glands from Day 18 of gestation. Staining is intense and diffusely distributed. G) Uterine gland from Day 18 of the estrous cycle. Staining is limited to a small area in the cytoplasm above the nucleus. Note the presence of large cells with clear cytoplasm within the glandular epithelium (arrows). H) Surface epithelium of the uterus on Day 18 of gestation. Positive staining is limited to the luminal surface of the epithelial cells. X1640.

A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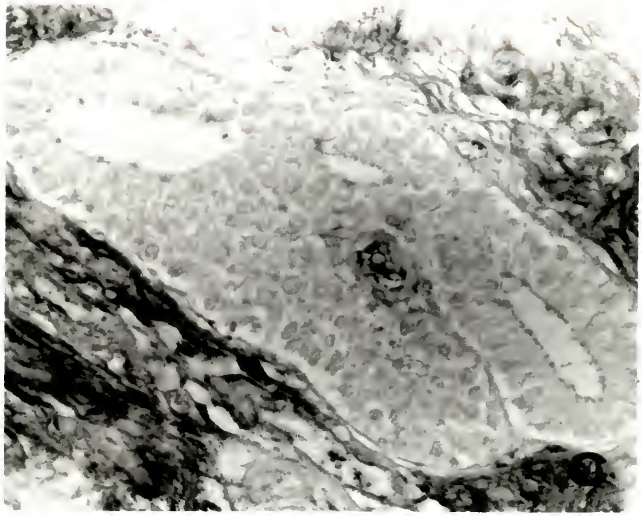

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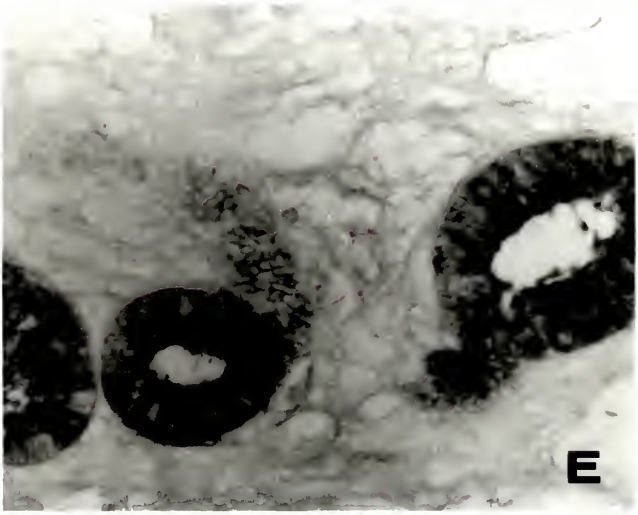
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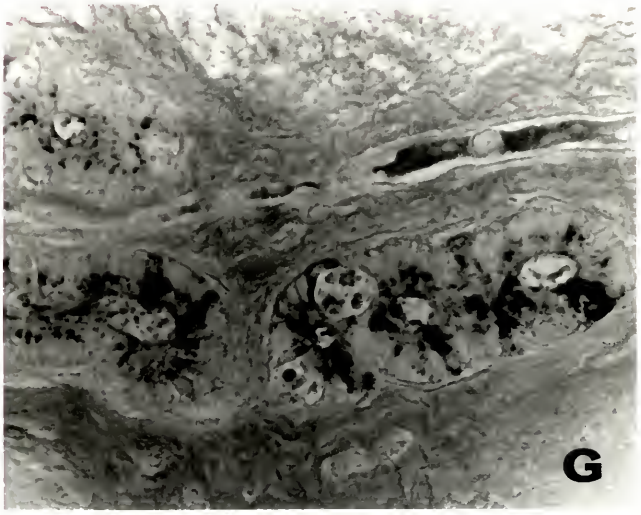
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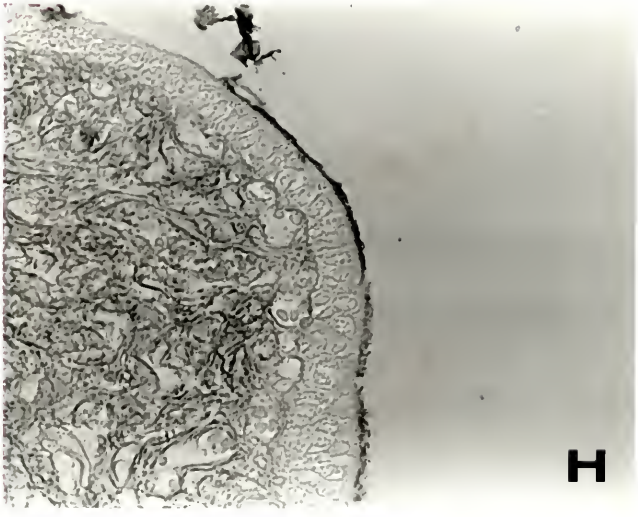

H

epithelium (Fig. 1.g); however, this staining may have resulted from adherence, during fixation, of uterine luminal secretory material containing Uf.

Endometrial Gland Ultrastructure and Acid Phosphatase Cytochemistry

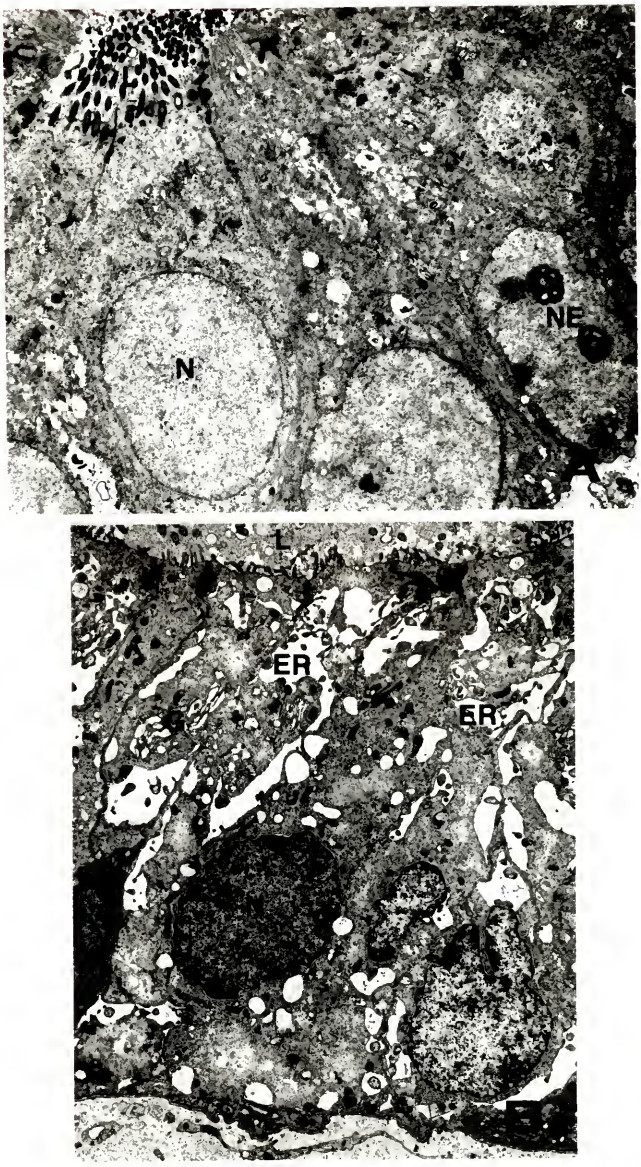
Glandular epithelium of endometrium taken from pregnant and nonpregnant gilts on Days 10, 12, 15 and 18 after the onset of estrus was examined. The ultrastructure of epithelial tight junctions, basement membranes, and organelles and structures involved in macromolecular synthesis and secretion were given special consideration. Acid phosphatase activity was used as an indirect probe for Uf localization. Uteroferrin is an acid phosphatase secreted by the uterine glandular epithelium, and it contributes 99% of the acid phosphatase activity measured in uterine flushings (Schlosnagle et al., 1974; Chen et al., 1975). Incubation medium used to localize acid phosphatase activity was prepared to give optimal conditions for acid phosphatase activity of Uf (p-nitrophenyl phosphate as substrate, incubation at pH 4.9) (Schlosnagle et al., 1974).

Ultrastructure and distribution of acid phosphatase in glandular epithelium was similar in tissues taken from pregnant and nonpregnant gilts on Days 10, 12 and 15 and will be described without regard to pregnancy status. Descriptions are limited to secretory (non-ciliated) cells of the basal portion of the glandular epithelium; however, descriptions of cells in the superficial glandular epithelium will be made when necessary for completeness.

Day 10 Postestrus

On Day 10 glandular epithelium was composed of tall columnar cells resting on a basement membrane approximately 78 nm in thickness (Fig. 3.2a). The apical plasma membrane was flat in most cells but concave in cells that appeared to be involved in secretion. In addition, short microvilli were present on cells with high secretory activity, while microvilli were longer and more numerous on less active cells. The lateral cell border was straight over most of its length except near the basal portion of the cell where apposed plasma membranes interdigitated. The apical portion of adjacent lateral cell membranes were joined by tight junctions (zona occludens) and several adhering junctions (zona adherens). The nucleus was round to oval in shape and located in the basal portion of the cytoplasm. In most cells the nuclear outline was regular with no indentations of the nuclear envelope. Within the nucleus, the chromatin was evenly distributed and one or two prominent nucleoli, with well marked differentiation between pars amorpha and nucleolonema (Fawcett, 1966), were usually present. The mitochondria were round to elongated in shape and appeared to be uniformly distributed within the cytoplasm. The mitochondrial matrix was dense, and the cristae were plate-like (lamellated). Highly elongated mitochondria, which extended nearly the entire length of the cell, were infrequently observed. The Golgi apparatus was located in the cytoplasm at the apical pole of the nucleus and each dictyosome of the Golgi was composed of 4 to 6 lamellae. Although the Golgi lamellae were not extensively swollen, the presence of numerous vesicles in close proximity to the lamellae indicated that the Golgi was active. These vesicles were 0.3 to 0.6 μ in diameter and frequently contained a

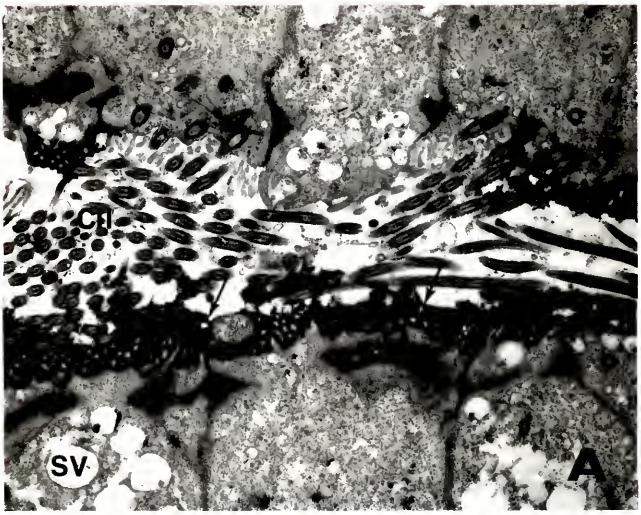
Figure 3.2. Transmission electron micrographs of glandular epithelium from a uterus on Day 10 of the estrous cycle. A) Epithelium of a gland in the basal area of the endometrium. The nucleus (N) is round and regular in outline with evenly dispersed chromatin and a prominent nucleolus (NE). Glandular lumen (L). X5600. B) Epithelium of a gland in the superficial area of the endometrium. Note the swollen ER cisternae. Golgi (G). X4600.


N
NE
L
ER
ER

fibrillar material, although absence of detectable content was not unusual. The apical cytoplasm contained numerous vesicles similar to those associated with the Golgi apparatus and these were occasionally seen to fuse with the apical plasma membrane (secretory vesicles). Their contents were similar to the fibrillar material observed in the glandular lumen. Fibrillar vesicles similar to those in the apical cytoplasm and lipid containing vesicles were infrequently observed in the basal cytoplasm beneath the nucleus. Tubular profiles of endoplasmic reticulum (ER) were present throughout the cytoplasm of most cells. However, secretory cells of superficial glands contained ER composed of swollen cisternae and vesicles (Fig. 3.2b). This form of ER was present in epithelial cells of superficial glands on all days examined. The cytoplasm of secretory cells was dense and granular in appearance, and glycogen deposits were not frequently observed.

Acid phosphatase activity was rarely associated with the luminal contents of the endometrial glands on Day 10; however, in some cells staining was associated with microvilli of the apical plasma membrane (Fig. 3.3a). The nuclear chromatin usually stained for acid phosphatase, but the pattern and intensity of staining varied among the cells examined. Acid phosphatase activity was infrequently observed in the Golgi lamella and, when present, the intensity of staining was low (Fig. 3.3b). In a few cells, staining was associated with the fibrillar contents of vesicles near the Golgi apparatus. Acid phosphatase staining was only rarely associated with apical secretory vesicles, and staining was not observed in vesicles in the basal portion of the cell. In most cells the endoplasmic reticulum did not appear to stain for acid phosphatase activity.

Figure 3.3. Transmission electron micrographs of acid phosphatase activity in glandular epithelium from a uterus on Day 10 of the estrous cycle. A) Acid phosphatase activity associated with microvilli (arrows) on the apical surface of the glandular epithelium. Cilia (Cil). Secretory vesicles (SV). X11,000. B) Acid phosphatase activity in the Golgi apparatus is sparsely distributed. Mitochondria (MT). X11,600.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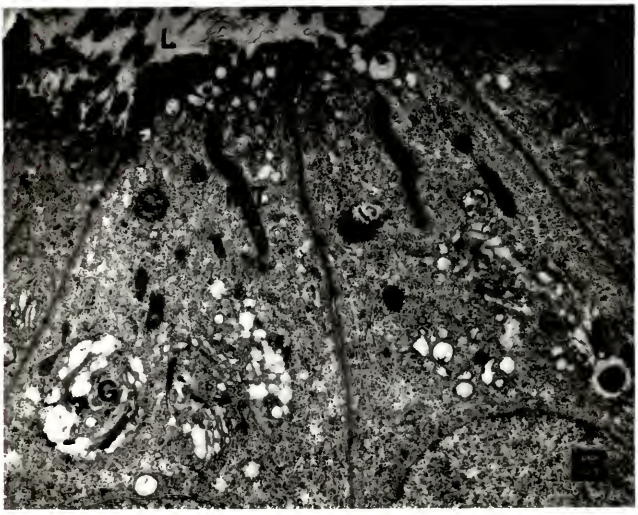
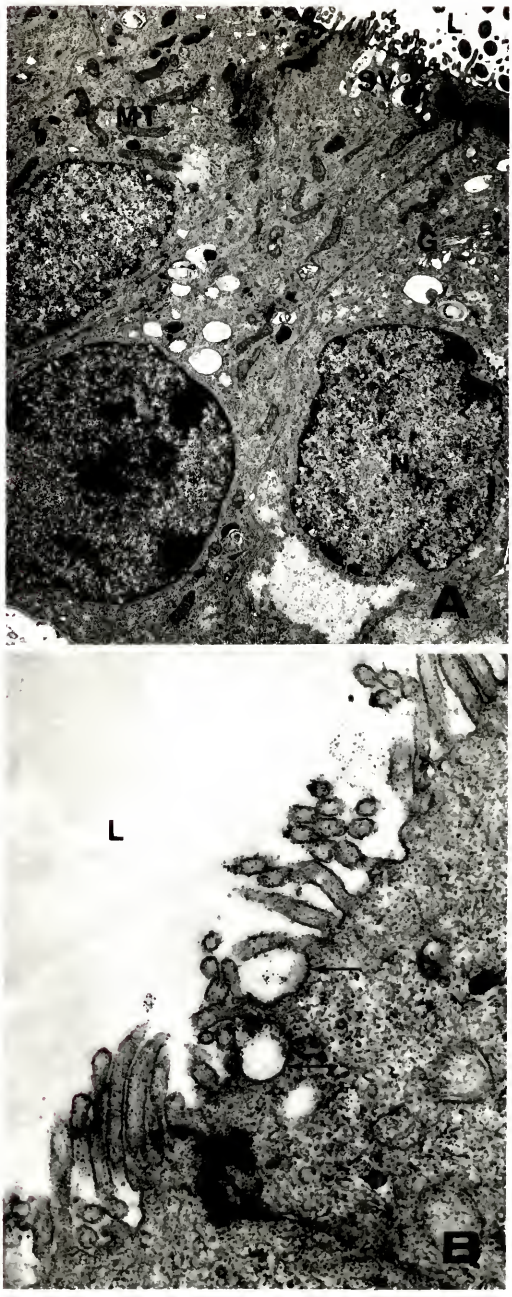

L
G

Day 12 Postestrus

On Day 12 cell shape and appearance of the cell borders were similar to Day 10. However, average width of the basement membrane increased ($P<0.05$) to 87 nM. Tight junctions and other intercellular junctions were present. The nucleus remained in the basal portion of the cell; however, its shape was variable with round, oval and elongated profiles observed (Fig. 3.4a). In addition the nuclear outline appeared to be irregular due to identations of the nuclear envelope. In contrast to Day 10, the chromatin in most cells was distributed in clumps throughout the nuclear compartment and heavy accumulations of chromatin were associated with the inner surface of the nuclear envelope. The nucleolus remained prominent in most cells. The ultrastructure of mitochondria was similar to that observed on Day 10; however, mitochondria were located primarily in the apical cytoplasm. The Golgi apparatus of Day 12 was similar in all respects to that described for Day 10. The number of secretory vesicles appeared to be increased compared with 10, and secretory profiles were more frequently observed (Fig. 3.4b). Basally located fibrillar vesicles were only occasionally observed; however, the number of lipid vesicles appeared to increase. The ER was abundant and located both sub- and supranuclear. Swollen supranuclear profiles of ER were frequently seen in cells of deep, as well as superficial, glands and subnuclear glycogen deposits were observed in most cells (Fig. 3.4c).

Acid phosphatase activity was frequently associated with the abundant apical microvilli; however, it was not possible to determine if staining was associated with the structure of the microvilli or with adhered secretory material. Acid phosphatase activity was associated with

Figure 3.4. Transmission electron micrographs of glandular epithelium from the uterus on Day 12 after the onset of estrus. A) Glandular epithelium from a nonpregnant gilt. X6500. Nuclear shape is variable with round and elongated profiles present. Note clumping of the chromatin within the nucleus and heavy accumulation associated with the nuclear envelope. The outline of the nucleus is irregular due to indentations of the nuclear envelope. B) Apical plasma membrane of a secretory cell in the glandular epithelium from a pregnant uterus. X30,400. Note the secretory vesicles that appear to have fused with the plasma membrane (arrows). C) Basal cytoplasm of glandular epithelium from a nonpregnant uterus. X5500. Glycogen deposits (Gl) are present in the cytoplasm beneath the nucleus.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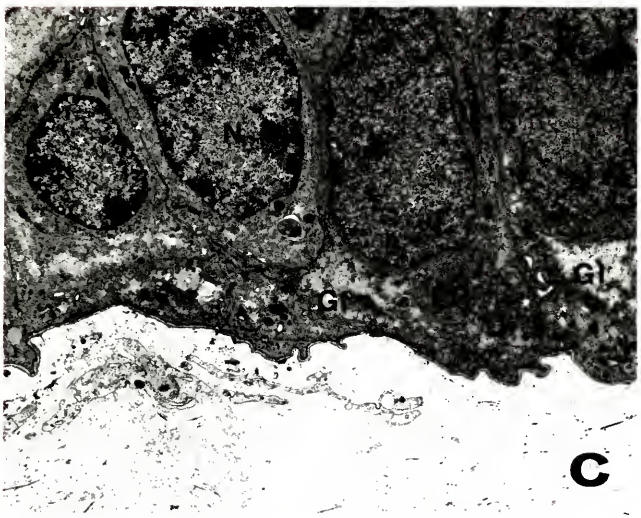
N
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Gl
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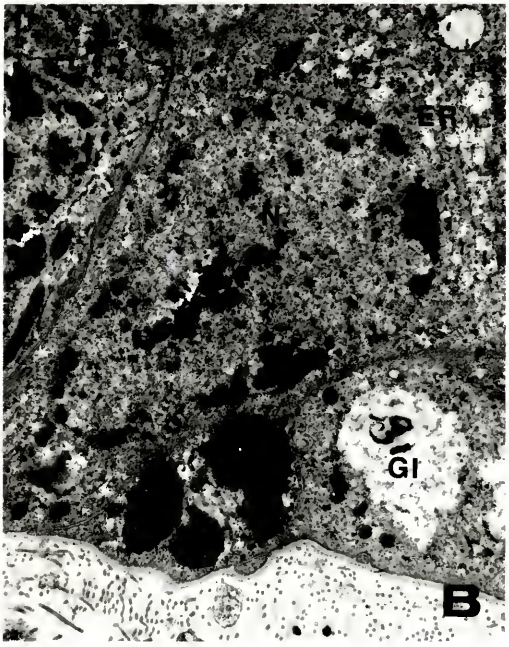
fibrillar material within the lumina of most of the glands examined (Fig. 3.5a). The nuclear chromatin was intensely stained in most of the cells examined and heavy accumulations were associated with clumps of chromatin (Fig. 3.5b). Acid phosphatase activity was present in the Golgi complex of most cells, and staining was frequently observed in vesicles near the Golgi appartus (Fig. 3.5c). The number of vesicles with acid phosphatase staining of their contents appeared to increase compared with Day 10 and these vesicles were frequently seen in close proximity to the apical plasma membrane (Fig. 3.5d). Acid phosphatase staining of the ER was highly variable. In some glands many cells contained acid phosphatase staining in the ER while in other glands only a few cells contained ER staining (Fig. 3.5e).

Day 15 Postestrus

Cell shape, appearance of the cell borders, number and structure of apical microvilli and intercellular junctions, and thickness of the basement membrane on Day 15 (Fig. 3.6) were similar to that observed on Day 12. The nucleus was elongated in shape and ireegular in outline in most cells, and the chromatin was heavily clumped. A distinct nucleolus with well differentiated pars amorpha and nucleolonema was not always present, and the frequency of its presence appeared to be reduced compared with Day 12. The ultrastructure and distribution of mitochondria and the ultrastructure of the Golgi complex and associated vesicles were similar to that described for Day 12. Apical secretory vesicles were numerous and these vesicles frequently appeared to fuse with the apical plasma membrane. Endoplasmic reticulum was abundant, and swollen supranuclear profiles were frequently observed. Subnuclear glycogen deposits and basal lipid vesicles were present in most cells.

Figure 3.5. Transmission electron micrographs of acid phosphatase activity in glandular epithelium from the uterus on Day 12 after the onset of estrus. A) Acid phosphatase activity associated with microvilli of the epithelium and luminal contents of a gland from a nonpregnant uterus. X9200. Note also the staining associated with the endoplasmic reticulum. B) Acid phosphatase staining associated with a nucleus in glandular epithelium from a nonpregnant uterus. X13,300. Note staining of the endoplasmic reticulum and presence of large lipid vesicles (LV). C) Acid phosphatase activity in the Golgi apparatus and associated vesicles of glandular epithelium from a pregnant uterus. X36,400. D) Acid phosphatase activity associated with the contents of a secretory vesicle in close proximity to the apical plasma membrane. X44,200. E) Acid phosphatase activity in the glandular epithelium of a nonpregnant uterus. X11,600. Note the variability in staining of the endoplasmic reticulum among the cells present.

L
ER
A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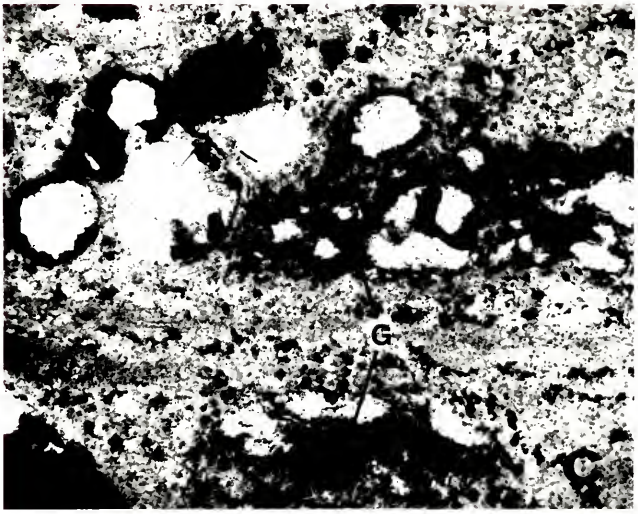
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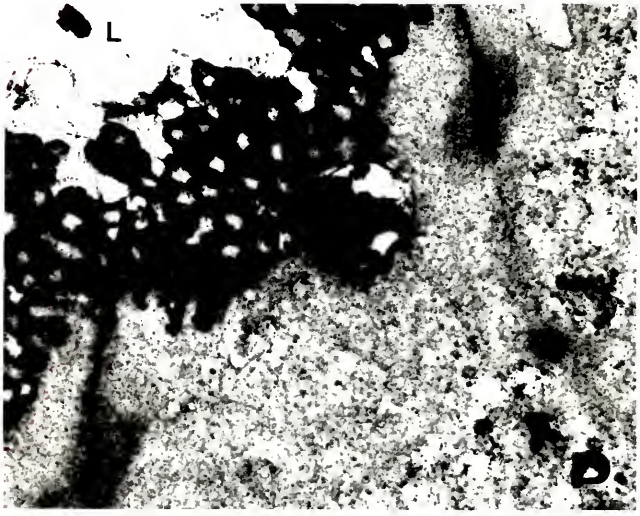
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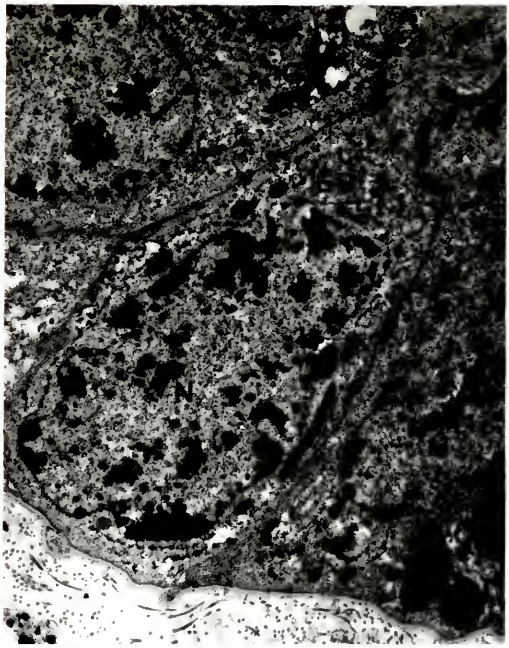
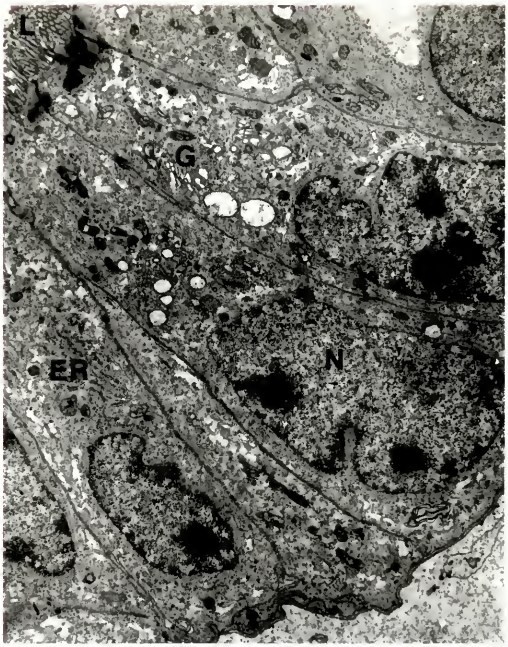


Figure 3.6. Transmission electron micrograph of the glandular epithelium from a uterus on Day 15 of gestation. X4900. Abundant endoplasmic reticulum and an active Golgi apparatus are present. The nucleus is elongated and irregular in outline, and the chromatin heavily clumped.


L
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N

Acid phosphatase was associated with luminal contents of most glands. In addition, staining was frequently observed on the outer surface of apical microvilli. Nuclear staining was observed in most cells. The Golgi cisternae in most cells contained heavy acid phosphatase staining which was not associated with a particular face of the complex. Acid phosphatase staining was frequently present in secretory vesicles with fibrillar contents which were located near the Golgi apparatus and within the apical cytoplasm. Apparent release of acid phosphatase stained secretory product into the lumen of the gland was observed in many cells. (Fig. 3.7a). Acid phosphatase staining in the ER was similar to that described for Day 12. Some glands contained cells with numerous acid phosphatase stained vesicles in the basal portion of the cell (Fig. 3.7b); however, most cells did not contain these vesicles.

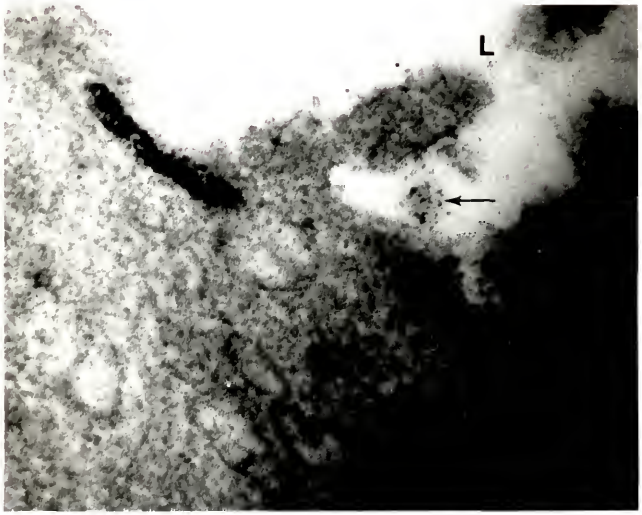
Day 18 of Gestation

The ultrastructure and acid phosphatase staining pattern of secretory cells on Day 18 of gestation (Fig. 3.8a,b) was similar in most aspects to that described for cells from Day 15 postestrus. However, basal vesicles containing lipid or acid phosphatase seemed to be observed more frequently.

Day 18 of the Estrous Cycle

The ultrastructure and acid phosphatase staining pattern of glandular epithelial cells on Day 18 of the estrous cycle was varied. A few cells resembled those from Day 18 of gestation. In many cells, the apical endoplasmic reticulum was extensively swollen and acid phosphatase staining was not present. In these same cells acid phosphatase was rarely

Figure 3.7. Transmission electron micrographs of acid phosphatase activity in glandular epithelium of the uterus on Day 15 after onset of estrus. A) Apical plasma membrane of a secretory cell in the glandular epithelium from a non-pregnant uterus. X156,000. Note apparent release of acid phosphatase stained secretory material into the glandular lumen (arrow). B) Acid phosphatase activity associated with vesicles in the basal cytoplasm (arrows) of glandular epithelium from a pregnant uterus. X24,700. Note the presence of lipid containing vesicles (LV).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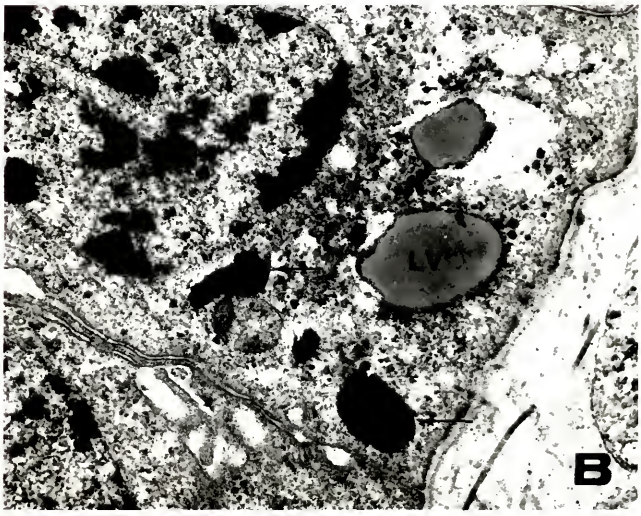
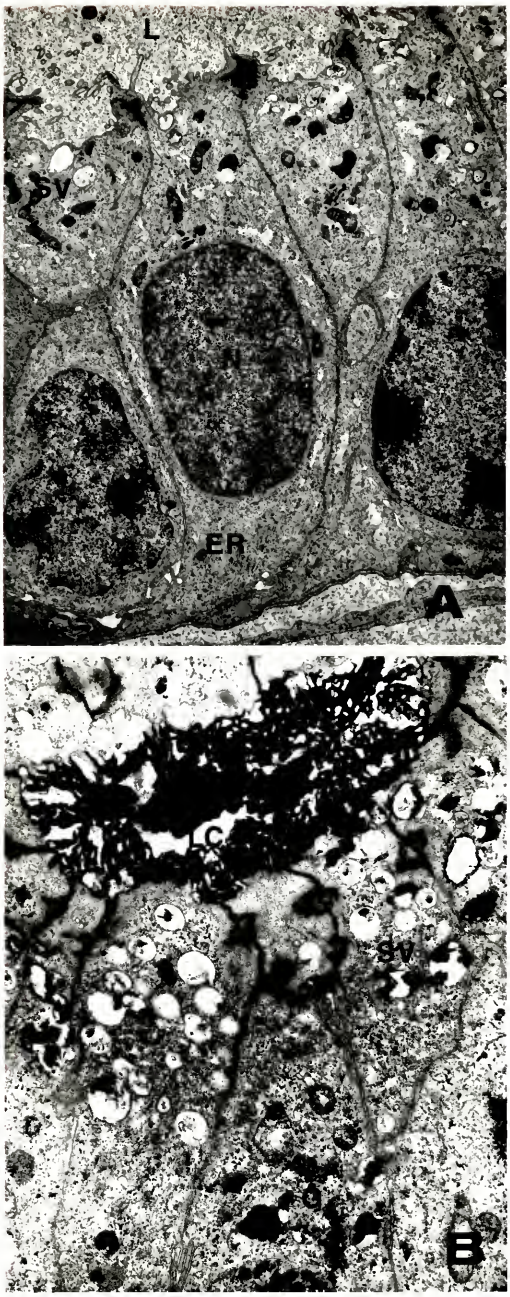

LV
B

Figure 3.8. Transmission electron micrographs of glandular epithelium on Day 18 of gestation. A) Typical morphology of the glandular epithelium. X7000. Apical secretory vesicles are numerous and luminal contents are abundant. B) Acid phosphatase activity in the apical portion of the glandular epithelium. X14,100. Note intense staining of luminal contents (LC) and frequent association of acid phosphatase activity with the contents of apical secretory vesicles.


L
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associated with the Golgi or apical secretory vesicles (Fig. 3.9a). Also, luminal contents were sparse and acid phosphatase staining was infrequent. In some glands the cells appeared shrunken and the cytoplasm was dark (Fig. 3.9a). The lateral borders between cells were not tightly adhered, with wide intercellular spaces present from the level of the nucleus to the base of the cell. Apical tight junctions were always present. At this stage of the estrous cycle large interepithelial cells were frequently observed (Fig. 3.9b). These cells were characterized by a light cytoplasm which contained numerous large electron dense vacuoles. The content of these vacuoles was not determined; however, acid phosphatase activity was present.

The lumina of superficial glands on all days examined frequently contained nuclei which suggested that holocrine secretion may be occurring. This was supported by one observation of a basal gland (Day 18 pregnant) in which cells appeared to be released into the glandular lumen (Fig. 3.10). However, the cytoplasm and nucleoplasm of these cells were electron lucent which may be indicative of a process for removal of degenerated cells.

The morphology and acid phosphatase distribution of uterine glandular epithelium observed in the present study were highly variable on each day of pregnancy or the estrous cycle. The descriptions presented represent that of a "typical" cell comprising the most common characteristics present on each day examined. Additional figures of the uterine glandular epithelium on Days 12 and 15 of pregnancy and the estrous cycle are presented in the Appendix to illustrate the variation in acid phosphatase activity. These days were chosen because maternal recognition of pregnancy occurs during this time.

Figure 3.9. Transmission electron micrographs of glandular epithelium on Day 18 of the estrous cycle. A) Shrunken cells with dark cytoplasm present in the glandular epithelium. X4700. Note the presence of wide intercellular spaces. B) Acid phosphatase activity associated with a large clear cell present in the glandular epithelium. X4400. Note intense staining of large dense granules within the cytoplasm (arrows). Acid phosphatase staining is also associated with lysosomes present in secretory cells of the glandular epithelium (arrowhead).

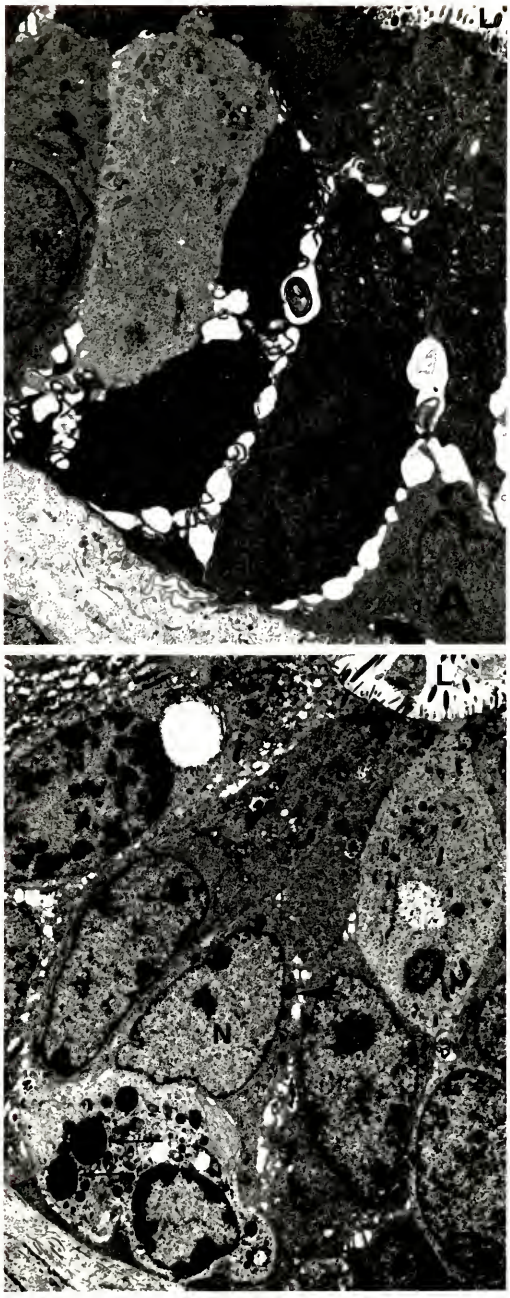
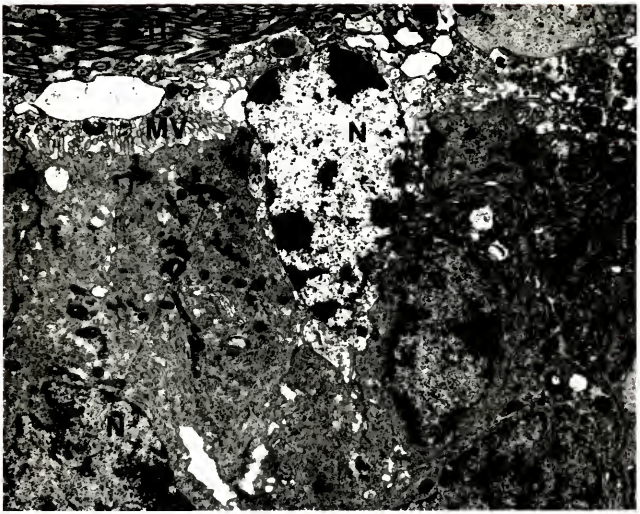

L
L
N

Figure 3.10. Transmission electron micrograph of the epithelium of a gland in the basal area of the endometrium from a uterus on Day 18 of gestation. X7000. Note the apparent release of an entire cell of the glandular epithelium into the glandular lumen. The cytoplasm and nucleoplasm of the released cell are relatively electron lucent. Note the presence of an apparently normal mitochondrion (arrow).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Collagenase-Like Activity

Endometrial CLA was measured to determine if changes in activity were associated with the shift from exocrine to endocrine release of uterine secretions (Day 12 to 15). Collagen is the primary structural component of basement membranes, and variations in associated CLA may alter the permeability of the basement membrane.

In the present study, active CLA increased ($P<0.05$) from 45.8 RU/mg protein on Day 8 to 143.4 RU/mg protein on Day 18 in nonpregnant gilts (Fig. 3.11). Mean active CLA in nonpregnant gilts was 89.0 ± 8.4 RU/mg protein. In pregnant gilts mean active CLA was 91.1 ± 8.4 RU/mg protein, but a day trend was not detected. Total endometrial CLA increased during the period examined for both pregnant and nonpregnant gilts ($P<0.01$); however, neither status nor status x day interaction effects were detected (Fig. 3.12). Total CLA averaged 114.1 ± 13.2 and 116.0 ± 13.0 RU/mg protein in pregnant and nonpregnant gilts, respectively, over the period studied.

Total and Free Hydroxyproline

Collagenase-like activity is inversely related to collagen content of the uterus in rats (Woessner, 1969; Ryan and Woessner, 1974), and total hydroxyproline (THP) is an estimate of tissue collagen content (Woessner, 1961). In addition, free hydroxyproline (FHP) in tissues arises from collagen degradation (Prockop and Kivirikko, 1968). Therefore, THP and FHP were determined in endometrial tissues as additional evidence of CLA.

Figure 3.11. Active collagenase-like activity (CLA) in endometrial tissue from pregnant (cross-hatched bar) and nonpregnant (open bar) gilts. Least squares means and standard errors are shown. Day effects ($P<0.05$) were detected for nonpregnant gilts. Collagenase-like activity was measured by incubating endometrial explants on ^{125}I-collagen gels for 3 hours at 37°C. One relative unit (RU) is 5% of the total ^{125}I-products released from gels by incubation with a standard collagenase preparation (4 mg/ml).

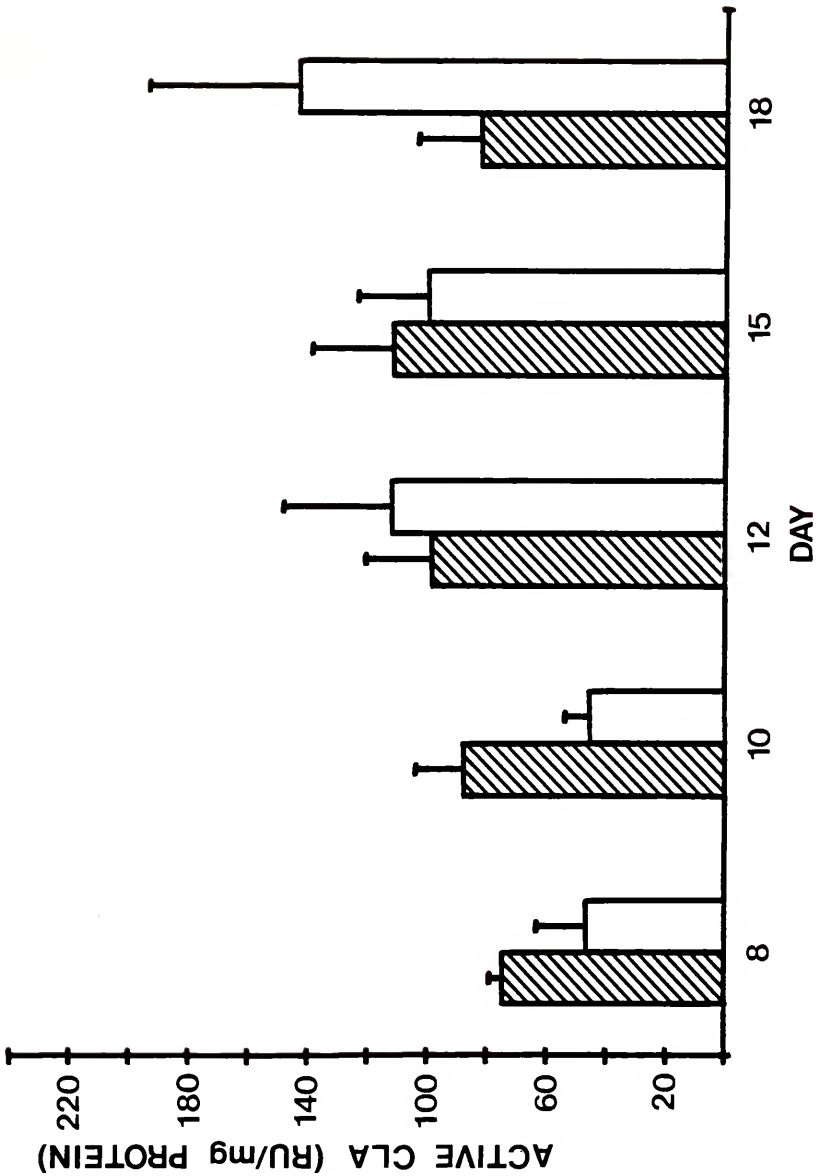
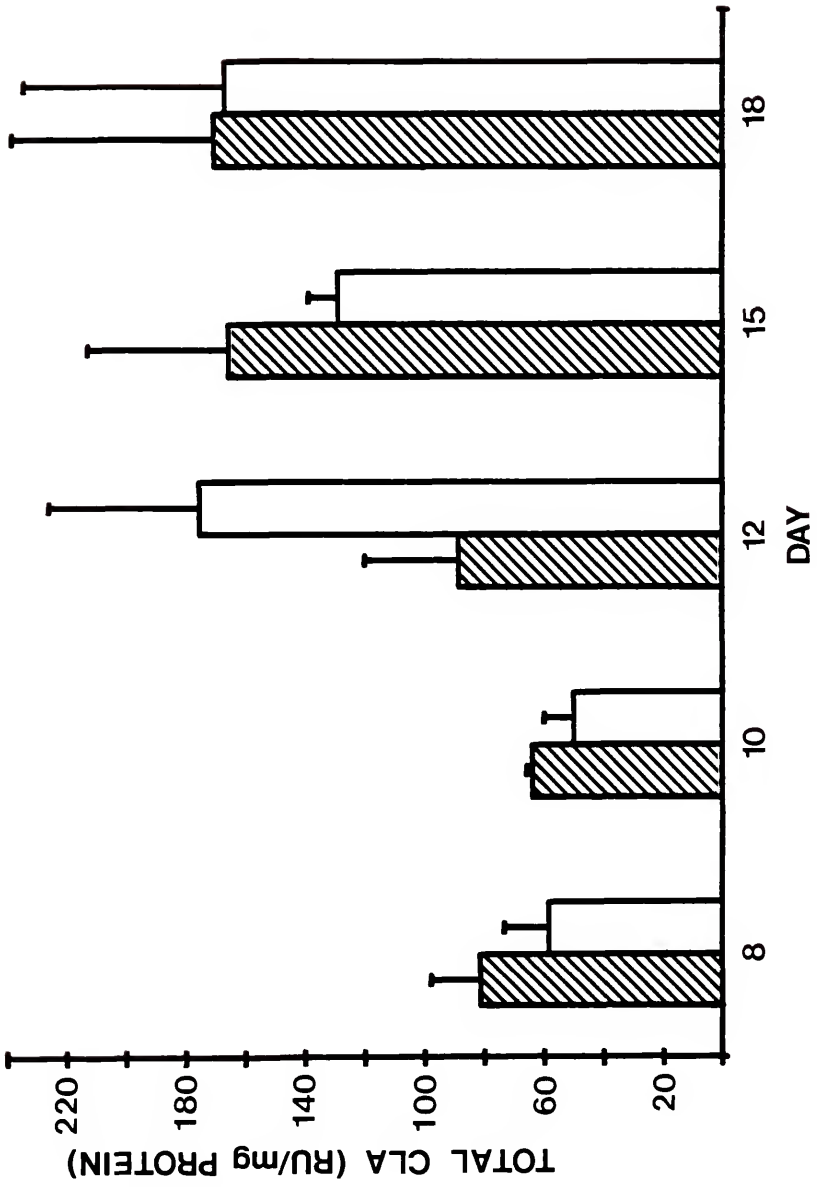

ACTIVE CLA (RU/mg PROTEIN)
220
180
140
100
60
20
8
10
12
15
18
DAY

Figure 3.12. Total collagenase-like activity (CLA) in endometrial tissue from pregnant (cross-hatched bar) and nonpregnant (open bar) gilts. Least squares means and standard errors are shown. Day effects ($P<0.01$) were detected for nonpregnant and pregnant gilts. Collagenase-like activity was measured in endometrial extracts previously treated with trypsin for 3 minutes to activate latent CLA. Activated extracts were incubated on ^{125}I-collagen gels for 3 hours at 37°C and activity was determined by the quantity of ^{125}I-products released. One relative unit (RU) is 5% of the total ^{125}I-products released from gels incubated with a standard collagenase preparation (4 mg/ml).


TOTAL CLA (RU/mg PROTEIN)
220
180
140
100
60
20
8
10
12
15
18
DAY

Total hydroxyproline decreased ($P<0.05$) between Days 8 to 12 and then increased ($P<0.03$) to Day 18 in both pregnant and nonpregnant gilts (Fig. 3.13). Neither status nor status x day interaction were significant. Free hydroxyproline (FHP) decreased ($P<0.001$) from Day 8 to 18 in pregnant gilts (Fig. 3.14). In nonpregnant gilts FHP decreased ($P<0.002$) between Days 8 and 12 and then increased ($P<0.002$) to Day 18. Free hydroxyproline averaged 55 and 173 μg/gm dry tissue on Day 18 of pregnancy and the estrous cycle, respectively.

Percent Dry Weight

Percent endometrial dry weight for both pregnant and nonpregnant gilts decreased between Days 8 and 18 (Fig. 3.15), but effects of status and status x day interaction were not significant.

Discussion

Chen et al. (1975) localized Uf in endometrial tissues from pregnant and nonpregnant gilts by immunofluorescence techniques. Uteroferrin was observed in the epithelial cells and lumina of uterine glands from Days 8 to 18 of early pregnancy. In nonpregnant gilts, Uf began to be localized within the stroma surrounding uterine glands between Days 12 and 15, and Chen et al. (1975) suggested that this change in Uf staining pattern may be indicative of a change from exocrine to endocrine release of uterine secretory products. A shift in the direction of release of Uf (exocrine to endocrine), as determined by the presence of stromal staining, was not observed in the present study when Uf was localized in endometrial tissues by immunoperoxidase techniques. Accordingly, plans to utilize this technique to examine the intracellular distribution of Uf by

Figure 3.13. Total hydroxyproline in endometrial tissue from pregnant (cross-hatched bar) and nonpregnant (open bar) gilts. Least squares means and standard errors are shown. Day effects were significant ($P<0.05$) for nonpregnant and pregnant gilts. Samples of endometrial tissue were hydrolyzed with 5 N HCl at 135°C, filtered and neutralized with NaOH. Portions of these solutions were assayed for hydroxyproline by "Method I" as described by Woessner (1961).

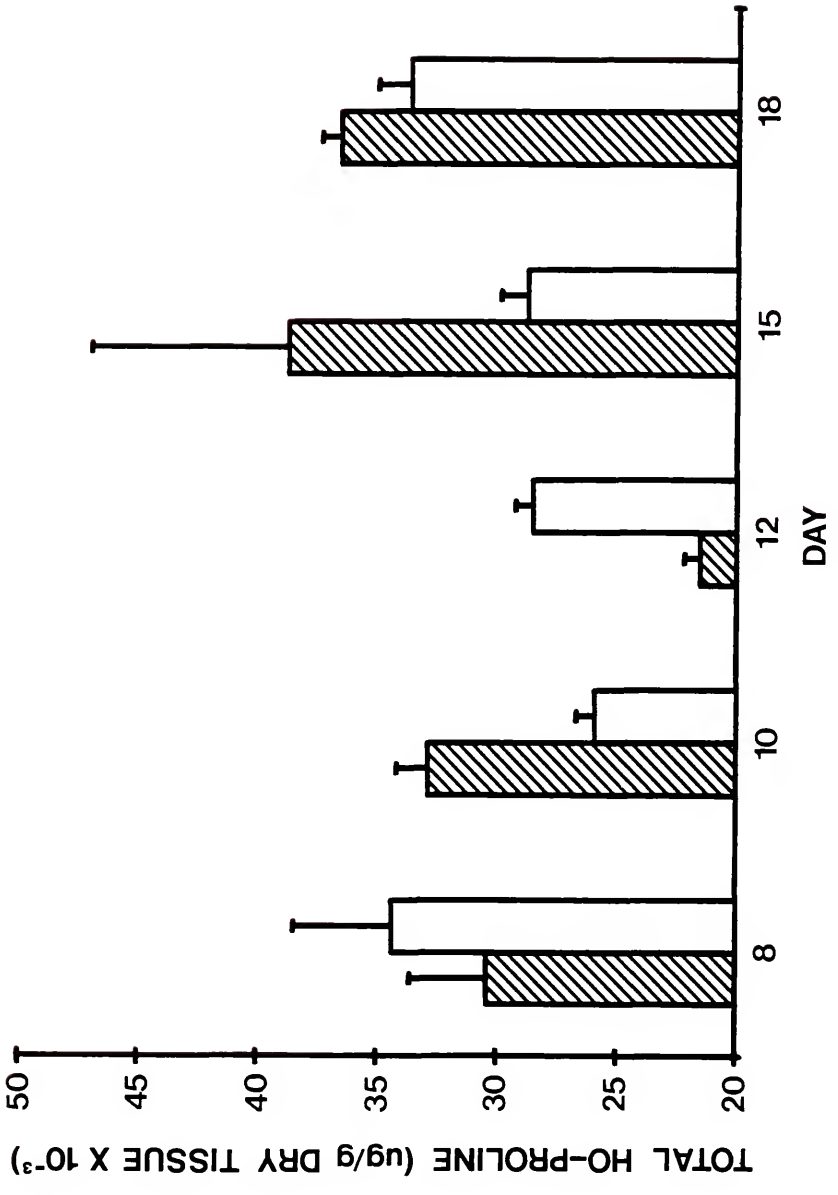

TOTAL HO-PROLINE (ug/g DRY TISSUE X 10⁻³)
50
45
40
35
30
25
20
8
10
12
15
18
DAY

Figure 3.14. Free hydroxyproline in endometrial tissue from pregnant (cross-hatched bar) and nonpregnant (open bar) gilts. Least squares means and standard errors are shown. Day effects were detected ($P<0.01$) for pregnant and nonpregnant gilts. Samples were prepared for free hydroxyproline determination by the method of Woessner (1969). Hydroxyproline was measured in prepared samples by "Method II" as described by Woessner (1961).

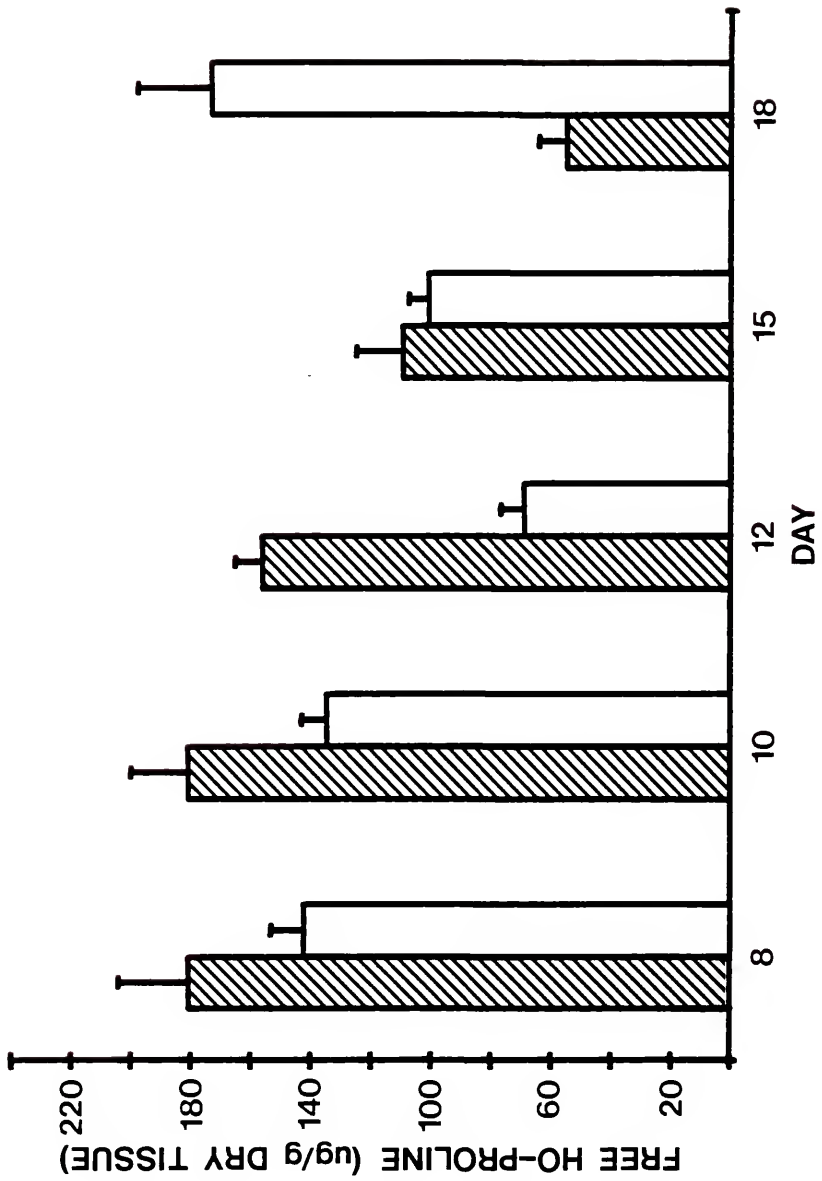
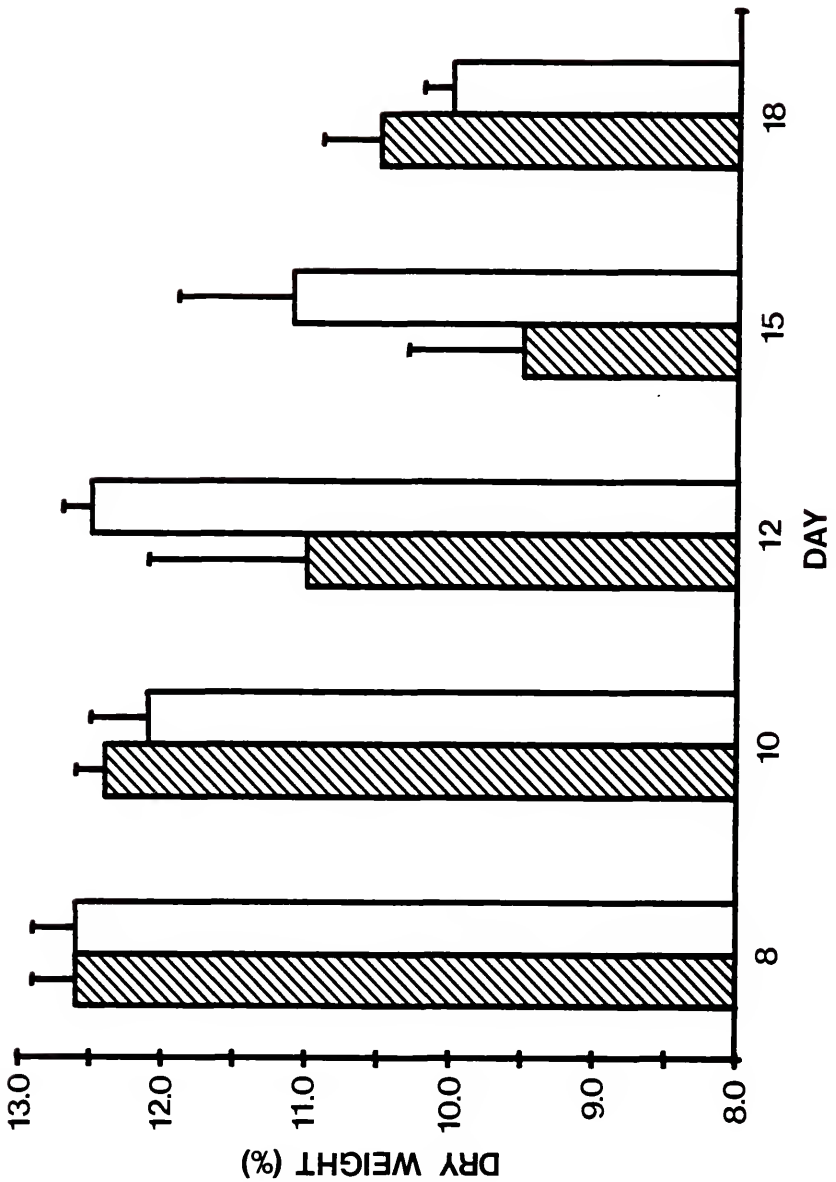

FREE HO-PROLINE (ug/g DRY TISSUE)
220
180
140
100
60
20
8
10
12
15
18
DAY

Figure 3.15. Percent dry weight of endometrial tissue from pregnant (cross-hatched bar) and nonpregnant (open bar) gilts. Least squares means and standard errors are shown. Day trends were significant ($P<0.01$) for nonpregnant and pregnant gilts. Dry tissue weight was determined by drying endometrial samples to constant weight in an oven at 80°C.


DRY WEIGHT (%)
13.0
12.0
11.0
10.0
9.0
8.0
8
10
12
15
18
DAY

electron microscopy, and to investigate the mechanism(s) responsible for a shift in the release of secretory products, were abandoned. The observations made during this study will be discussed later. Stromal tissue surrounding endometrial glands is composed primarily of collagen fibers and amorphous ground substance. A few fibroblasts are also present. Inability to detect Uf in the stroma on Day 15 in the present study may reflect nonspecific removal of Uf by the techniques used to prepared tissues for immunoperoxidase localization. Chen et al. (1975) localized Uf in tissues prepared by freezing and sectioning on a cryostat. Presumably, this technique of tissue preparation would not cause extensive loss of extracellular materials. However, removal of stromal Uf by fixation procedures used in the present study is not likely since Uf was observed to remain in the lumina of glands.

There was no evidence of a change in the ultrastructure of glandular epithelial cell tight junctions between Days 12 and 15 of the estrous cycle as would be expected if tight junctions were involved in the shift in release of secretory product from exocrine to endocrine. Tight junctions appeared to remain intact on all days examined, and this fact together with the inability to detect intercellular acid phosphatase activity indicated that Uf does not enter the stroma by movement from the glandular lumen between cells of the epithelium.

Likewise, continuity, density and thickness of the basement membrane were not different on Day 15 of the estrous cycle when compared with Day 12, which suggested that alterations of the basement membrane are not involved in regulating the direction of uterine secretion. Direct evidence of hormonally induced changes in epithelial basement membranes are not available. However, Armstrong et al. (1973) reported that the

basement membrane of human glandular epithelium was approximately 30 nM in width during the proliferative phase of the menstrual cycle when plasma estrogen concentrations are increasing and progesterone concentrations are low. Thickness of the basement membrane increased during the secretory phase when progesterone concentrations are high and estrogen concentrations are low. The increase in basement membrane thickness between Days 10 and 12 in the present study may represent the final stage of an increase which began after ovulation and formation of the CL.

Vesicles with fibrillar contents, similar to apical secretory vesicles, were occasionally observed in the basal portion of epithelial cells; however, their appearance was not associated with a particular day of the estrous cycle or gestation. Basal vesicles that contained acid phosphatase activity were increased between Days 12 and 15, but this increase was observed in both pregnant and nonpregnant gilts. Release of fibrillar material or material with acid phosphatase activity by secretory vesicles at the basal plasma membrane was not detected in any of the tissues examined. These observations suggest that release of uterine secretory products at the basal plasma membrane is not responsible for a shift in the direction of release of uterine secretory products during the period of maternal pregnancy recognition. The increase in basal acid phosphatase staining vesicles between Days 12 and 15 probably indicated an increase in the number of basally located lysosomes, rather than Uf secretory vesicles, since the number of basal dense vesicles appeared to increase in unstained tissues. The function of increased basal lysosomes in pregnant gilts is not known; however, in nonpregnant animals they may have a role in uterine changes which occur after regression of the CL in preparation for return to estrus (Corner, 1921).

Differences in the pattern of change of endometrial active and total CLA between pregnant and nonpregnant gilts were not associated with the time of maternal recognition of pregnancy (Days 12 to 15) which suggested that CLA mediated alterations of the basement membrane were not responsible for a shift in release of uterine secretory products. However, measurement of CLA by the described procedure would not differentiate between CLA associated with the basement membrane and that associated with stromal collagen fibers. Therefore, definitive conclusions as to the role of CLA in basement membrane alterations are not possible.

Hormonal control of uterine collagenase activity in vivo has been examined (Woessner, 1969; Ryan and Woessner, 1974; Woessner, 1979). Woessner (1969) reported that collagen degradation in the uteri of postpartum rats was inhibited by daily injections of estradiol 17β. Subsequently, Ryan and Woessner (1974) and Woessner (1969) demonstrated that decreased collagen breakdown in postpartum rats given estradiol was associated with decreased uterine collagenase activity. Inhibitory effects of other steroid hormones on in vivo collagenase activity have not been examined; however, Jeffrey et al. (1971) demonstrated that collagenase production by postpartum uterine cultures was abolished by addition of 50 μM progesterone to the culture medium. In addition, the inhibitory effect of progesterone increased when estradiol was present in the culture medium. Estradiol alone did not inhibit collagenase production by uterine cultures. These results led Ryan and Woessner (1974) to suggest that in vivo inhibition of uterine collagenase may not be mediated by direct action of estradiol on the uterus. However, Cartwright et al. (1977) found complete inhibition of collagenase release

from cultured uterine explants by estradiol at a concentration of 50 μM. These data indicate that steroid control of uterine collagenase production is not well understood.

In the present study total CLA increased in pregnant and nonpregnant gilts in association with the period when peripheral plasma progesterone concentrations are reported to increase (Day 4-12) (Guthrie et al., 1972). Two possible reasons for this association may be that progesterone stimulates the production of CLA by the endometrium or that the collagenolytic activity measured was not the same as that measured by Woessner (1979). The latter is most likely since collagenolytic activity measured in this study was not inhibited by removal of Ca from the assay media as was the case in the study by Woessner (1979).

In pregnant gilts, active CLA did not change during the period studied. Collagenase breakdown of collagen is necessary for tissue remodeling and the constant level of activity observed in the endometrium of pregnant gilts may reflect rapid uterine growth occurring at this time (Perry and Rowlands, 1962). Increasing active CLA in nonpregnant gilts between Days 8 and 15 may also be associated with uterine changes in preparation for pregnancy. The further increase in activity between Days 15 and 18 probably reflects collagenolytic activity associated with the involution process occurring in the porcine uterus during proestrus.

Uterine collagen and FHP were variable during the period studied and changes in their concentrations were not related to previously reported variations in the concentrations of ovarian steroids (Guthrie et al., 1972). These data are in contrast to the report of Smith and Kaltreider (1963) which indicated that progesterone was stimulatory to uterine collagen accumulation in rats. Also, changes in uterine collagen and FHP

were not associated with observed changes in collagenolytic activity which suggested that these measurements are not adequate for indirect determination of collagenolytic activity. High uterine collagen concentrations and low FHP concentrations on Day 18 of pregnancy are probably indicative of uterine growth associated with establishment of pregnancy. High FHP concentrations on Day 18 of the estrous cycle may reflect degradative processes occurring during uterine involution.

The decline in uterine endometrial dry weight in pregnant gilts during the period studied probably reflects the action of estradiol produced by the early blastocyst. In nonpregnant gilts, ovarian estradiol production, which increases between Days 14 and 16 of the estrous cycle (Guthrie et al., 1972), may contribute to decreased uterine dry weight on Days 15 and 18. In both cases, the estrogens may enhance tissue water imbibition.

Although identification of the cellular mechanism responsible for regulation of exocrine to endocrine release of uterine secretory products was not determined, detailed information was obtained regarding synthesis and secretion by cells of the uterine glands.

Roberts et al. (1982) examined the Uf secretory capacity of endometrial explant cultures established with tissues taken from nonpregnant gilts on various days of the estrous cycle. Explants established early in the cycle produced no Uf initially, but did so later in the culture period. In addition, the later in the cycle that explants were established the more Uf was produced. These authors (Roberts et al., 1982) suggested that the secretory capacity of an explant is determined by the number of cells present with the potential to secrete Uf. This was confirmed in the present study. Localization of Uf in the epithelium

of uterine glands by immunoperoxidase techniques indicated that Uf was first not present (Day 8), then present in some cells of some glands (Days 10 and 12) and finally present in all cells of all glands (Day 15). Uteroferrin was not observed in the lumina of uterine glands until Day 12 which corresponds to the day of the estrous cycle when it is first detected in uterine secretions (Murray et al., 1972). In addition, decreased localization of Uf between Days 15 and 18 of the estrous cycle corresponds to the time (Day 16) when Uf is no longer detected in uterine secretions (Murray et al., 1972).

All tissues examined, with the possible exception of those taken on Day 18 of the estrous cycle, were probably subject to high plasma progesterone concentrations at the time of collection. This is supported by the fact that the ultrastructure of the secretory cells of the glandular epithelium was similar to that of the glandular epithelium of cows during the luteal phase of the estrous cycle (Kojima and Selander, 1970) and of humans during the secretory phase of the menstrual cycle (Cavazos et al., 1967; Armstrong et al., 1973). Murray et al. (1972) reported that total protein content of uterine secretion from nonpregnant gilts was increased between Days 12 and 15 after the onset of estrus. In the present study several morphological changes in the secretory epithelium were associated with this period of increased protein secretion. These changes included increased release of secretory vesicles at the apical plasma membrane and increased frequency of ER profiles with swollen and vesiculated cisternae. Also, on Day 10, the nucleus was ovoid in shape and regular in outline with chromatin content being predominantly diffusely distributed. On Day 12, the nucleus was more elongated in shape, had an irregular outline and the chromatin was in a partially condensed form. Frenster (1969)

suggested that diffusely arranged chromatin indicated a high level of active transcription while clumped chromatin indicated that a large portion of the chromatin is inactive with regard to transcription. Increased clumping of nuclear chromatin, increased numbers of secretory vesicles and increased activity of the ER on Day 12 is in keeping with a cell that is expending most of its energy for synthesis and secretion of proteins. Thus final stages of differentiation to a secretory-type epithelium appeared to occur between Days 10 and 12 after the onset of estrus.

Prominent glycogen deposits and basal lipid droplets in uterine glandular epithelial cells are characteristic of the secretory phase of the estrous (Kojima and Selander, 1970) and menstrual (Cavazos et al., 1967; Armstrong et al., 1973) cycles. Boshier and Holloway (1973) reported that histochemical localization of neutral lipids in the uterine epithelium of sheep was increased by progesterone treatment which suggests that progesterone secretion by the CL stimulated appearance of basal lipids in the present study.

On Day 18 of the estrous cycle shrunken cells with dark cytoplasm and wide intercellular spaces were frequently observed. Similar descriptions are reported for gland cells from the late secretory phase of the menstrual cycle (Cavazos et al., 1967; Armstrong et al., 1973) and these changes are presumably indicative of uterine modifications in preparation for return to estrus.

Padykula and Taylor (1975) examined remodelling of the endometrium of the North American opossum at the end of diestrus and postpartum. These authors reported that stromal ground substance was removed via engulfment by macrophages which then migrated through the uterine

glandular epithelium into the lumen of the gland. Basally located intraepithelial cells observed in the present study on Day 18 (Fig. 3.1g and 3.9b) may be macrophages active in this process.

Electron microscopic localization of acid phosphatase activity was utilized as an indirect probe for Uf. Acid phosphatase activity associated with the Golgi apparatus, ER, secretory vesicles, apical microvilli or luminal contents was infrequently observed on Day 10, but staining in all of these areas increased on Day 12. These observations suggested that synthesis and secretion of Uf was increased during this period and that these processes proceeded by the generally accepted mechanisms (Palade, 1975). Based on acid phosphatase activity, Uf synthesis and secretion continued to Day 18 of pregnancy in agreement with previous biochemical estimations of Uf in the uterine lumen. Acid phosphatase staining was reduced in the Golgi apparatus, ER and secretory vesicles on Day 18 of the estrous cycle indicative of decreased Uf synthesis and release, which is in agreement with previous determinations of Uf within uterine flushings.

Zamiri and Blackshaw (1979) examined acid phosphatase activity in the endometrium of sheep at different times during the estrous cycle and reported that acid phosphatase activity in the uterine glands was weak during most of the estrous cycle. To the best of the authors' knowledge, ultrastructural localization of acid phosphatase in the endometrial glands has not been reported for any other domestic species. However, Hoffman and DiPietro (1972) examined the subcellular localization of acid phosphatases in human endometrial and placental tissues taken during the first trimester of pregnancy and determined that activity was present only in lysosomes of the glandular epithelium. This

observation together with the close agreement of acid phosphatase activity and previous estimates of Uf secretion (Murray et al., 1972) suggests that Uf contributes a portion of the acid phosphatase activity which was observed in pig endometrial epithelium.

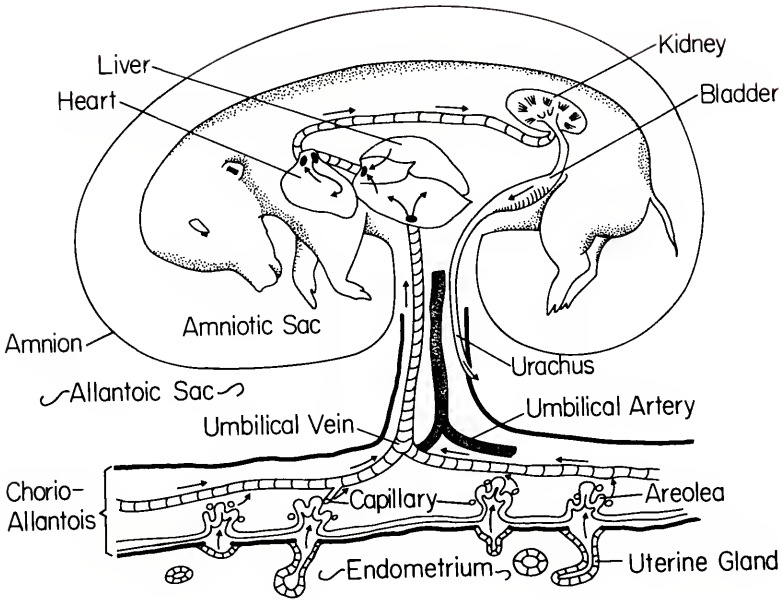
In summary, these data indicate that changes in intercellular tight junctions, the basement membrane and cellular secretory processes could not be used to explain the exocrine to endocrine shift in the direction of release of uterine secretory products. Histological and histochemical observations of the uterine glands during the estrous cycle and early pregnancy support previously reported cyclic patterns of uterine protein and Uf secretion which were determined by measurement of the protein content of uterine flushings. Finally, these data demonstrate maintenance of endometrial secretory activity associated with continued CL function following maternal pregnancy recognition.

CHAPTER IV
PLACENTAL TRANSPORT AND DISTRIBUTION OF UTEROFERRIN IN THE FETAL PIG

Introduction

Establishment of an epitheliochorial placenta in the pig (Grosser, 1909) and a number of other animals including the horse, camel and whale (Amoroso, 1952) causes little or no destruction of either the uterine mucosa on the maternal side or chorionic epithelium on the fetal side. Final attachment of the conceptus is achieved by interdigitation of epithelial microvilli and not by invasive growth (Brambel, 1933). The blood supply of mother and fetus are therefore separated by several tissue layers, and macromolecules required for embryonic growth and development are secreted by the glandular endometrium of the uterus. These secretions are absorbed by the chorion at specialized cup-shaped regions called areolae which form opposite the uterine glands (Fig. 4.1; Amoroso, 1952; Chen et al., 1975). The absorptive function of the chorionic areolae was recently examined using transmission electron microscopy by Friess et al. (1981). The authors observed numerous coated vesicles and tubules in the apical cytoplasm immediately beneath the microvilli of the areolar epithelium. The content of most of these vesicles appeared similar in electron density to substances within the areolar lumen, supporting the suggestion that secretions were being actively absorbed for use by the conceptus. In a recent review, Roberts and Bazer (1980) discussed uteroferrin (Uf), a progesterone-induced basic glycoprotein secreted by

Figure 4.1. Proposed route of Uf transport from the uterus to the fetoplacental unit. Uteroferrin secreted by the uterine glands is pinocytosed by cells of the areolae, released into the chorioallantoic capillaries and transported to the fetus via the umbilical vein. Within the fetus, Uf is bound by the liver, presumably to supply iron for hematopoiesis. Uteroferrin not removed by the liver is partially cleared by the kidney, accumulates within the bladder, and enters the allantoic sac in urine released through the urachus.


Liver
Heart
Kidney
Bladder
Amniotic Sac
Amnion
Allantoic Sac
Urachus
Umbilical Vein
Umbilical Artery
Chorio-
Allantois
Capillary
Areolea
Endometrium
Uterine Gland

the uterine glandular epithelium of the pig, which contains one molecule of iron (Fe) and possesses an acid phosphatase activity that is enhanced by reducing agents (Murray et al., 1972; Schlosnagle et al., 1974; Buhi et al., 1982b). Uteroferrin is produced in large amounts, particularly in midpregnancy when synthesis may exceed 1 g/day (Basha et al., 1979), and is believed to function in Fe transport from mother to fetoplacental unit (Roberts and Bazer, 1980; Buhi et al., 1982a; Ducsay et al., 1982). Since allantoic fluid of midpregnant gilts also contains Uf (Bazer et al., 1975) of maternal origin (Ducsay et al., 1982), it must be transported intact across the chorioallantois (placenta). It has been suggested that Uf-mediated Fe transport to the fetus is by direct passage across the chorioallantois into allantoic fluid from where Fe is distributed to the fetus. In support of this, Buhi et al. (1982a) demonstrated that the Fe on Uf in allantoic fluid was rapidly transferred to transferrin which could gain access to the fetal circulation and transport Fe to centers of hematopoiesis, particularly the liver (Ducsay et al., 1982). However, other data indicated that, although Fe in the allantoic sac is in a dynamic state, accumulation of Fe within allantoic fluid does not precede transfer of Fe to fetal tissues (Ducsay, 1980). This study was designed to examine critically Uf transport across the placenta and determine its subsequent distribution within the fetoplacental unit.

Materials and Methods

Animals and Surgical Procedures

Gilts with two previous estrous cycles of normal duration (18-22 Days) were checked daily for estrus with intact boars and bred when

estrus was detected and at 12 and 24 h after detection of estrus. Surgical procedures were performed with gilts under general anesthesia and tissues were obtained after exposure of the reproductive tract by midventral laparotomy (Knight et al., 1977).

Experiment 1.

Uterine endometrium with apposed chorioallantois was obtained from gilts on Days 60, 75, 90 and 105 of pregnancy (n=4, one gilt/day) to localize Uf by immunohistochemical staining. Tissues were prepared, and Uf was localized by the peroxidase-antiperoxidase bridge (PAP) technique as described in Chapter III.

Experiment 2.

Immunoreactive Uf was measured in fetal (n=17) umbilical arterial (UA) and venous (UV) plasma by a double antibody radioimmunoassay (RIA) procedure. Two gilts were laparotomized on Day 75 and the uterus exposed to allow exteriorization of each fetus through an incision in the adjacent antimesometrial wall of the uterus, chorioallantois and amnion. Blood samples (5 ml) collected from an umbilical artery and the umbilical vein in heparinized syringes (100 IU/sample) were chilled and centrifuged at 7700 x g to obtain plasma which was stored at -20°C until assayed.

The RIA was performed with the same sheep antiserum to Uf which was described in Chapter III (Immunocytochemical Localization of Uteroferrin). The assay buffer contained 20 mM barbital, 0.9% (w/v) NaCl, 0.029% (w/v) EDTA and 0.25% (w/v) bovine serum albumin (BSA) and was adjusted to pH 8.0 with 1.0 N hydrochloric acid. Tracer (^{125}I-Uf) was prepared by the Iodo-Gen technique (Markwell and Fox, 1978; Markwell, 1978) using the

procedure of Buhi et al. (1982a). Uteroferrin was assayed in 0.1 ml samples of fetal plasma to which 0.1 ml assay buffer was added. Standards (0.1 ml) dissolved in assay buffer received 0.1 ml of hysterectomized gilt plasma. Buffer (0.4 ml) and the specific antiserum (0.1 ml; 1:10,000 dilution) were then added and the assay held at 4°C for 24 h. In a series of prior experiments it was established that this dilution of antiserum precipitated 30% of the tracer in 0.1 ml of ^{125}I-Uf. Following the addition of ^{125}I-Uf (0.1 ml; 20,000 cpm) the assay was incubated for an additional 24 h. Next, sheep γ-globulin (0.1 ml; 400 μg/ml; ICN Nutritional Biochemicals, Cleveland, OH) and rabbit antibody raised against sheep γ-globulin (0.1 ml; 1:3 dilution; Antibodies Inc., Davis, CA) were added, and the assay incubated 48 h. After incubation, tubes were centrifuged at 2250 x g for 20 min, washed with cold assay buffer, recentrifuged and the pellet counted in a γ-counter. The specific antiserum did not crossreact with transferrin (2 mg/ml) or lactoferrin (1 mg/ml). Addition of 2 and 50 ng of Uf yielded values ($\bar{X}$ ± S.E.M.) of 2.0 ± .3 ng (n=7) and 50.3 ± 3.8 ng (n=4), respectively. The working range of the assay was from 1-2 ng to 50-60 ng and there was no evidence of nonparallelism between the standard curve and a curve produced by adding an increasing volume of a plasma sample containing purified Uf ($Y = -3.03X_1 + 7.31$ vs $Y = -2.71X_2 + 4.31$ where X_1 = log of volume and X_2 = log of standard curve dose). The minimum detectable value that was different from 0 was determined to be 0.5 ng during validation of the assay. Intra- and interassay coefficients of variation were 10.4 and 7.1%, respectively.

Data were analyzed by least squares analysis of variance to determine effects of gilt, fetuses within gilt, source of sample (umbilical artery or vein) and gilt by source of sample interaction.

Experiment 3.

In a preliminary study, Uf binding by fetal liver membranes was examined. Livers were removed from fetuses obtained by hysterectomy of gilts on Day 75 of pregnancy in Experiment 2. Crude liver plasma membrane was prepared by a modification of the method of Ray (1970). Tissues were minced in cold saline (0.9% (w/v) NaCl) and washed several times to remove blood. Samples of blotted tissue (~5g) were homogenized in a Dounce tissue homogenizer (0.64-1.40 mm clearance; Wheaton, Millville, NJ) with 40 ml of homogenization buffer (0.5 M $CaCl_2$-1 mM $NaHCO_3$, pH 7.5) until most cells were broken, but nuclei remained intact. All homogenization and storage solutions contained 1 mM protease inhibitor (phenylmethylsulfonyl fluoride; Sigma Chemical Co., St. Louis, MO). The homogenate was diluted to 500 ml in homogenization buffer and held on ice for 5 min to allow lysing of unbroken cells. This solution was then filtered through cheesecloth and centrifuged at 1500 x g for 30 min (GSA rotor, Dupont Instruments-Sorvall, Newtown, CT). The supernatant fraction was aspirated taking care not be disturb the buff-colored membrane layer above the pellet. The total pellet was resuspended by homogenization, diluted to 250 ml and centrifuged at 1500 x g for 15 min. Aspiration, resuspension, dilution (150 ml) and centrifugation were repeated. After removal of the final supernatant, the buff-colored membrane layer was gently washed from the pellet and stored in sucrose (0.25 M) at -20°C.

For liver binding analyses, isolated membranes (in 0.25 M sucrose) were centrifuged at 27,100 x g (SS-34 rotor; Dupont Instruments-Sorvall, Newtown, CT) for 20 min and the pellet retained for the assay and protein

determination. To determine membrane protein, 0.5 ml of the isolated membranes in 0.25 M sucrose plus 1.5 ml of 1 N NaOH were placed in a boiling water bath and shaken until clear. Protein was quantified in this solution by the method of Lowry et al. (1951) using BSA as the standard. A membrane stock solution was prepared by adding a volume of assay buffer (25 mM Tris-HCl, 10 mM $CaCl_2$, 0.1% BSA, pH 7.6) to the pellet to give 3 mg membrane protein/ml. In the Uf binding assay each tube contained ^{125}I-Uf (20,000 cpm) and liver membrane (75-600 µg protein) brought to a total volume of 0.5 ml with assay buffer (all tubes were duplicated). Competitive displacement of bound tracer was accomplished by adding radioinert Uf (2.5-400 µg). Assays were incubated for 90 min at 25°C in 1 ml polypropylene centrifuge tubes. After incubation, tubes were centrifuged (Eppendorf Microfuge, Brinkmann Instrument, Inc., Westbury, NY) for 4 min. The pellet obtained was washed with buffer, recentrifuged and the final pellet counted.

Experiment 4.

Urine samples were collected from Day 75 fetuses (n=27) obtained at laparotomy from gilts in Experiments 2 and 5. Fetuses were exteriorized as previously described, umbilical cords tied and severed, and the abdominal wall opened to expose the bladder. Urine samples were taken with a 5 ml syringe fitted with a 22 gauge needle and stored at -20°C until analysis. Protein concentration was determined by the method of Lowry et al. (1951) using BSA as standard. Acid phosphatase activity was measured in sample aliquots treated with β-mercaptoethanol (Schlosnagle et al., 1974; Buhi et al., 1982b). Following acid phosphatase analysis, samples were randomly assigned to agar gel double immunodiffusion

analysis (n=8) or polyacrylamide gel electrophoresis (n=3). This was necessary as small sample volumes and low protein content prevented analysis by both techniques. For immunodiffusion (Ouchterlony, 1958). urine samples were concentrated by lyophilizing 1 ml aliquots and resuspending the sample in 0.1 ml of PBS. Urine concentrates (10 µl) or Uf standard were applied to wells in the agar (0.1% (w/v) Noble agar, DIFCO Laboratories, Detroit, MI) of immunodiffusion plates and developed against antiserum to Uf at room temperature in a humidified chamber. Plates were photographed without prior staining. For 2D-PAGE, urine sample aliquots containing 200 to 450 µg protein were lyophilized and the dried material dissolved in 150 µl of 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol (Horst and Roberts, 1979). Samples were electrofocused by the nonequilibrium pH gradient electrophoresis technique (NEPHGE) for basic proteins as described by Basha et al. (1980). Protein migration was toward the cathode, and the duration of electrofocusing was 3.5 h. Sodium dodecylsulfate-gel electrophoresis in the second dimension was performed by the method of Basha et al. (1980) and Laemmli (1970). Slabs were fixed and stained with Coomassie blue R-250 before photography.

Experiment 5.

Two pregnant gilts were hysterectomized on Day 75 and fetal liver and kidney tissue obtained to localize Uf by immunocytochemical staining. Liver tissue was fixed by immersion in the fixative as described in Chapter III (Immunoperoxidase Localization of Uteroferrin). Kidneys were perfused with fixative via the renal artery until the tissue blanched.

Kidneys were subsequently bisected and fixation continued by immersion. Uteroferrin was localized by the PAP procedure described in Chapter III.

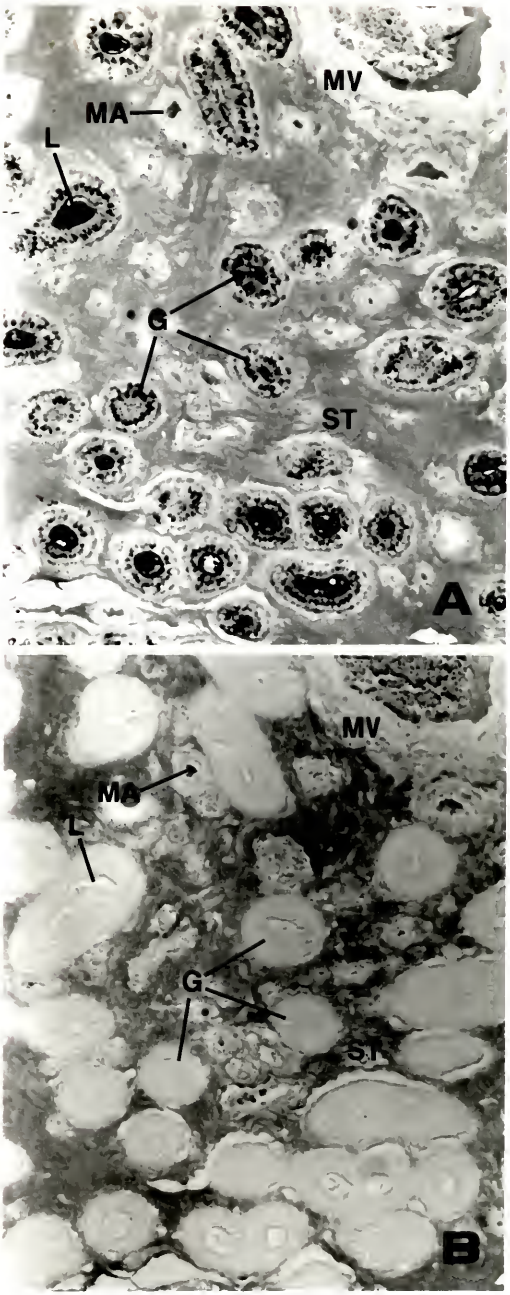
Results

Experiment 1.

The relationship between the maternal uterine endometrium and fetoplacental unit is shown in Fig. 4.1. Positive Uf staining was observed in the lumen and epithelial cells of the uterine glands on Days 60, 75, 90 and 105 of pregnancy. In Fig. 4.2 endometrium from a Day 60 animal is shown, and this staining pattern was present on all days examined. Within the glandular epithelium Uf appeared localized primarily in the apical cytoplasm above the large clear nucleus. The stromal cells and maternal blood vessels did not stain. Staining was present in endometrial surface epithelial cells adjacent to the chorionic areolae, but not in other cells of the surface epithelium (Fig. 4.2c).

When chorionic tissues were examined, cells of the areolae could be distinguished by their large size and content of vesicles. The latter stained intensely for Uf (Fig. 4.3a). Staining was concentrated in large and small vesicles located in both the supra-and infranuclear cytoplasm of the cell (Fig. 4.3a). In particular, there were high concentrations of infranuclear vesicles located in cells immediately adjacent to chorioallantoic capillaries (Fig. 4.3b). In some sections positive staining was seen at the border of capillaries and in some instances appeared to be in the process of being released from cells of the areolae into a capillary (Fig. 4.3c). This observation is consistent with the work of Ducsay et al. (1982) which demonstrated uptake of ^{59}Fe-Uf by placental

Figure 4.2. Uterine endometrium from a gilt on Day 60 of pregnancy stained for Uf by the peroxidase-antiperoxidase method. A) Staining for Uf was present in the apical cytoplasm of endometrial epithelial cells of the uterine glands (G) and within the lumina (L) of the glands. Staining was not present in maternal arteries (MA) or veins (MV) or within the endometrial stroma (ST). X575. B) Adjacent section to the section shown in Plate A. The peroxidase-antiperoxidase method was performed with normal sheep serum in place of antiserum to Uf. Positive staining is not present. X575. C) Surface epithelium of the endometrium (E) contained staining for Uf (arrows) in cells adjacent to the chorioallantoic areolae (AR) but not in cells of the surface epithelium outside the areolae. Hematoxylin counter stained. X1600.


MV
MA
L
G
ST
A
MV
MA
L
G
ST
B

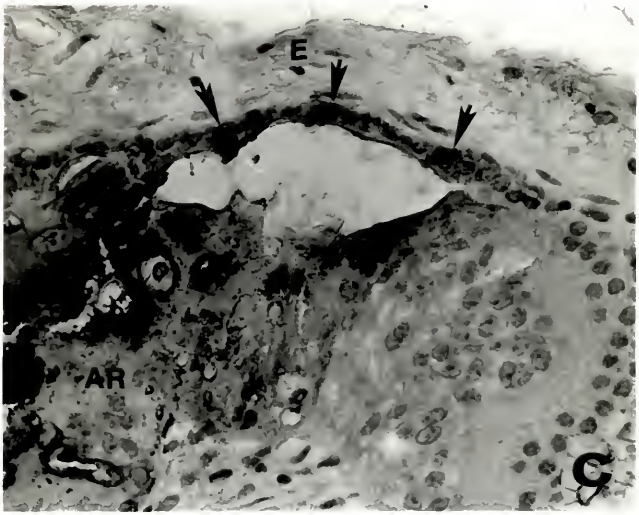
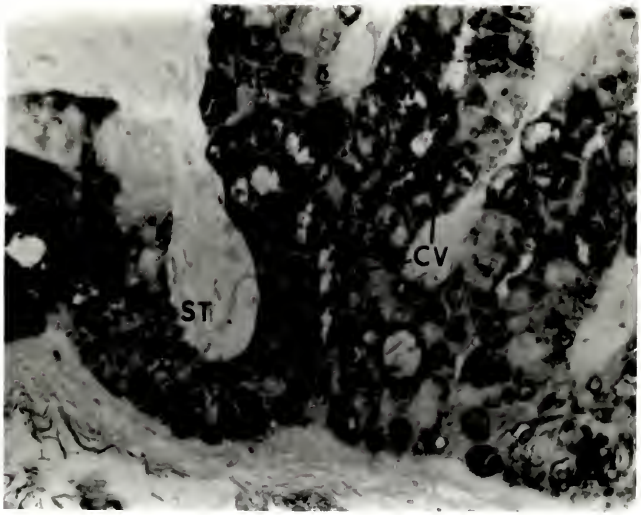
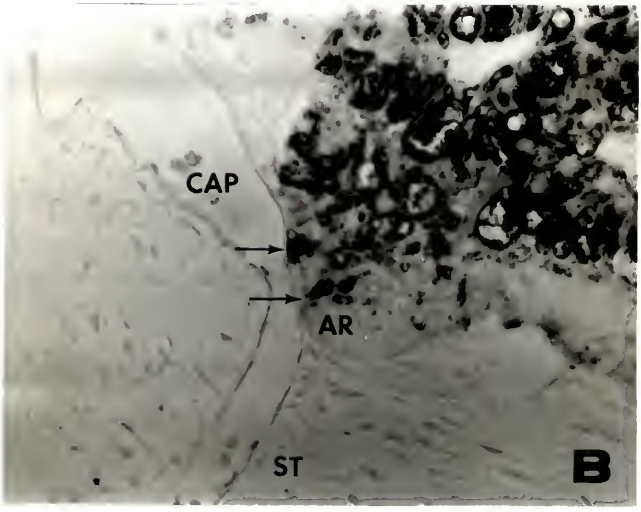
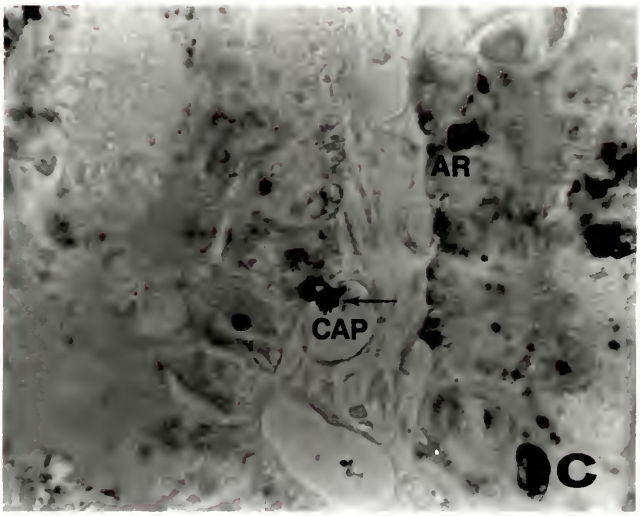

E
AR
C

Figure 4.3. Peroxidase-antiperoxidase staining for Uf in Day 75 placental tissue. A) Positive staining in cells of the chorioallantoic areolae (AR) was present within supra- and infranuclear cytoplasmic vesicles (CV). Note the absence of staining within the surrounding chorioallantoic stroma (ST). X1060. B) Section of an areola with Uf staining in infranuclear cytoplasmic vesicles (arrows) of cells adjacent to a chorioallantoic capillary (CAP). X1200. C) Section of an areola showing apparent release of Uf (arrow) from infranuclear cytoplasmic vesicles into a chorioallantoic capillary (CAP). X1400.


CV
ST


CAP
AR
ST
B


AR
CAP
C

areolae and subsequent transport to the fetal circulation. Positive staining for Uf was not observed in the mesenchymal tissue of the chorioallantois, epithelial cells of the allantois or chorionic epithelial cells outside the areolae. The described staining pattern was present on all days examined and control experiments confirmed the specificity of staining for Uf.

Experiment 2.

Immunoreactive Uf was detected in plasma from all Day 75 fetuses in low concentrations. However, there was considerable variation in plasma Uf concentrations among different fetuses. Mean Uf concentrations in umbilical venous plasma was 80.0 ± 26.0 ng/ml and ranged from 29.0 to 450.0 ng/ml at Day 75 of pregnancy. Umbilical arterial plasma Uf concentrations were less ($P<0.07$) than those for umbilical venous plasma and averaged 44.0 ± 13.0 ng/ml with a range of 20.0 to 155.0 ng/ml. The mean venous-arterial Uf concentration difference among 17 fetuses was 36.0 ± 18.0 ng/ml with 13 positive values (range 1.5 to 298.0 ng/ml) and 4 negative values (-2.3 to -7.8 ng/ml). These data indicate that maternal Uf gains access to the fetal blood and that a portion of it is removed from the circulation during passage through fetal tissue.

Experiment 3.

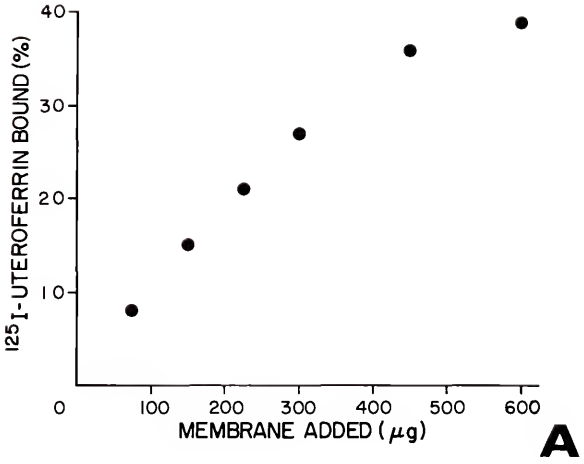
Earlier work (Buhi et al., 1982a) showed that ^{59}Fe from Uf is sequestered mainly in the liver. To determine whether fetal liver membranes bound Uf, an assay was conducted with the addition of increasing amounts of membrane protein to constant amounts of labeled Uf in the absence of unlabeled Uf. The proportion of radiolabeled Uf bound

increased from 7.7% of total counts added in the presence of 75 µg of membrane protein up to 39.2% with 600 µg of membrane protein (Fig. 4.4a). Incubation of a constant amount of membrane protein (100 µg) and radiolabeled Uf with increasing amounts of purified radioinert Uf (2.5-400 µg) substantially decreased binding compared with that in the absence of unlabeled Uf (Fig. 4.4b). These data are from a single experiment. This experiment was not designed to characterize the Uf receptor but to determine whether cell membranes of liver tissue have the ability to bind Uf in a dose-dependent manner. These data, although of a preliminary nature, support that possibility.

Experiment 4.

Urine samples obtained from the bladders of Day 75 fetuses contained 189.0 ± 15.9 µg protein/ml. Acid phosphatase activity was detected in urine of 10 of 27 fetuses and specific activity averaged 3.37 ± 1.83 µmol inorganic phosphate (Pi) released/10 min/mg protein. Values ranged from a high of 19.3 units/mg protein to a low of 0.4. Three samples of eight tested gave a discernible line of identity when tested against purified Uf using sheep antiserum against Uf by Ouchterlony double immunodiffusion analysis (Fig. 4.5). Two-dimensional NEPHGE analysis of urine identified a protein with electrophoretic mobility similar to Uf in one individual and one pooled urine sample (Fig. 4.6) while in another individual sample Uf was not detected. Pooling was necessary to obtain enough protein for electrophoresis. Interestingly, several other polypeptides with mobilities similar to other basic proteins found in maternal uterine secretions (Basha et al., 1980) were also detectable in fetal urine (Fig. 4.5,

Figure 4.4. Day 75 fetal liver membrane binding of ^{125}I-uteroferrin. A) Addition of increasing quantity of membrane protein (75-600 µg). Membranes and ^{125}I-uteroferrin (20,000 cpm) were incubated at 20°C for 90 min. B) Addition of increasing quantity of unlabeled Uf (2.5-400 µg). A constant quantity of membrane protein was used (150 µg). Values are expressed as percentage of the binding in control tubes which did not contain unlabeled Uf (% B_0).


40
30
20
10
0
100
200
300
400
500
600
^{125}I-UTEROFERRIN BOUND (%)
MEMBRANE ADDED (μg)
A

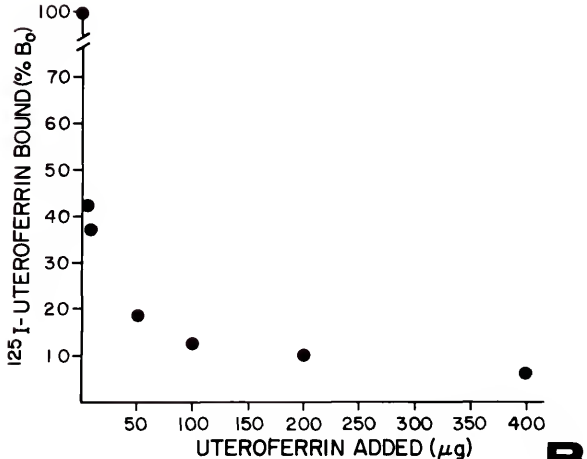

100
70
60
50
40
30
20
10
50
100
150
200
250
300
350
400
^{125}I-UTEROFERRIN BOUND (% B_0)
UTEROFERRIN ADDED (μg)
B

Figure 4.5. Agar gel double immunodiffusion of a Day 75 fetal urine sample (U) and purified Uf against sheep antiserum to Uf in the center well.

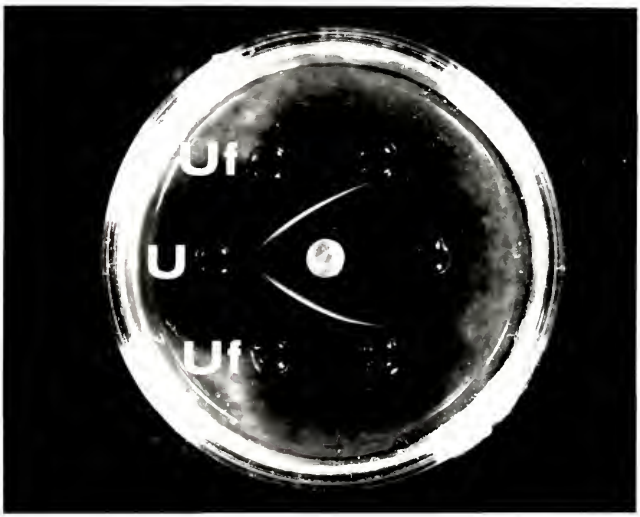
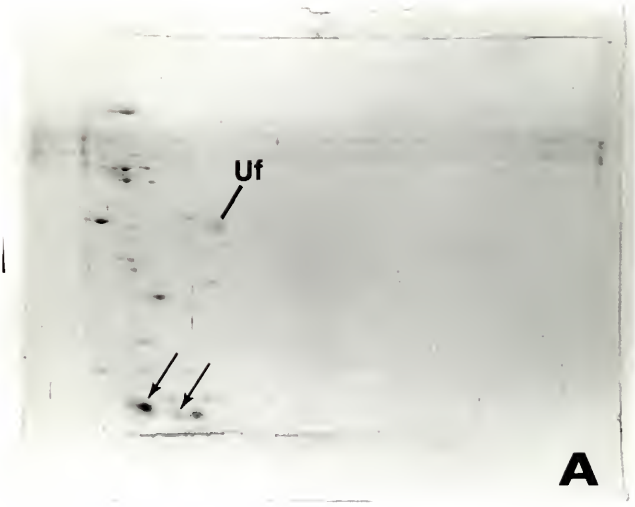

Uf
U
Uf

Figure 4.6. Coomassie blue stained two-dimensional polyacrylamide gel electrophoresis of basic proteins (NEPHGE). A) A urine sample from a Day 75 fetus. Uteroferrin (Uf) and two other proteins (arrows) similar to those found in uterine secretions were present. B) Uterine secretions from Day 11 of pregnancy.


Uf
A

Uf
B

arrows). These data strongly suggest that fetal urine contains small, but highly variable amounts of Uf and possibly other proteins found in endometrial secretions.

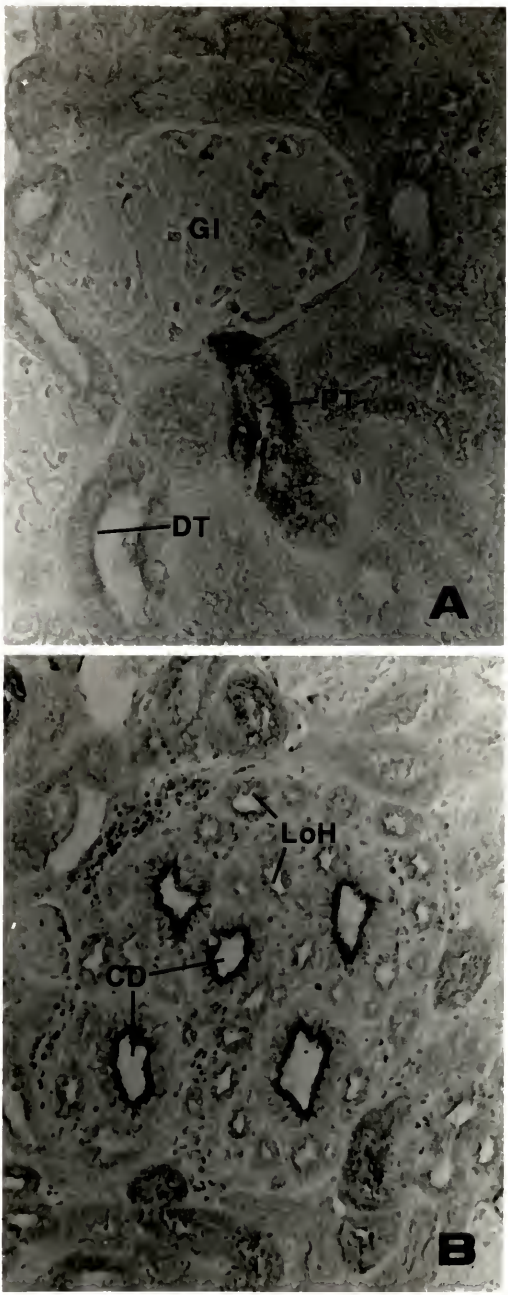
Experiment 5.

Weak immunocytochemical staining for Uf was observed within the epithelial cells and lumen of proximal tubules (Fig. 4.7a) and within the lumen of collecting ducts (Fig. 4.7b) of Day 75 fetal kidney tissue. Only a few cells within each proximal tubule contained positive Uf staining. Staining in these cells may indicate Uf uptake by proximal tubules which is consistent with the fact that proximal tubules are active in absorption of proteins from the glomerular ultrafiltrate (Guyton, 1976). In collecting ducts, staining was restricted to the periphery of the lumen and this staining pattern probably resulted from concentration and adherence of protein to the luminal wall. Not all kidney tissues tested had positive staining and among tissues there appeared to be variations in relative staining intensities. Positive staining for Uf was not detected within distal tubules or within tubules of the Loop of Henle in kidney tissue. At this time there is no explanation for the absence of staining in some kidneys or the variation in staining intensity observed. Uteroferrin was not detected in fetal liver by the PAP procedure.

Discussion

Immunohistochemical localization of Uf in the uterine glandular epithelium and lumen is consistent with previous data which suggested that these cells are the site of synthesis and secretion of this protein.

Figure 4.7. Peroxidase-antiperoxidase staining for Uf in Day 75 fetal kidney tissue. A) Cortical area of the fetal kidney. Positive staining is present in the lumen and epithelial cells of the proximal tubules (PT) but not within distal tubules (DT) or glomerulus (GI). X1600. B) Cross-section of a medullary ray with positive staining present along the walls of the collecting ducts (CD). Positive staining was not observed in tubules of the Loop of Henle (LoH). X650.


GI
PT
DT
A
LoH
CD
B

Basha et al. (1979) reported Uf production by endometrium in tissue culture, and Chen et al. (1975) demonstrated by immunofluorescent antibody techniques that Uf was localized only in cells of the glandular and surface epithelium. In the present study staining was observed only in surface epithelial cells adjacent to the chorioallantoic areolae suggesting that the surface epithelium is not a major site of Uf synthesis and secretion. Staining of the surface epithelium adjacent to the areolae may indicate the presence of an intermediate cell type having the morphological characteristics of surface epithelium but the biosynthetic capacity to secrete Uf. In support of this Crombie (1972) reported that uterine epithelial cells adjacent to the areolae were ultrastructurally different from interareolar uterine epithelium. In addition, Friess et al. (1981) observed large blebs budding from the apices of uterine epithelial cells bordering the areolar lumen which were not observed on uterine surface epithelium outside the areolae. Wislocki and Dempsey (1946) examined the pig placenta for Fe and acid phosphatase activity by histochemical techniques and found little reaction within the uterine surface epithelium, but abundant localization within uterine glands. Since Uf appears to be the major placental Fe transport protein in pigs (Ducsay, 1980; Roberts and Bazer, 1980; Buhi et al., 1982a) and possesses >95% of the acid phosphatase activity in uterine secretions (Bazer et al., 1975), the data of Wislocki and Dempsey (1946) in conjunction with that from the present study suggest that surface epithelium has little or no role in Uf secretion. It is possible that the fluorescence within the surface epithelium observed by Chen et al. (1975) was autofluorescence which has been reported in this tissue by Wislocki and Dempsey (1946).

In the chorioallantois, Uf was localized in the lumen and cells of the areolae, but not other areas of the tissue. Chen et al. (1975) also demonstrated Uf in areolae, and Wislocki and Dempsey (1946) observed abundant iron and acid phosphatase activity in these structures. In the present study Uf was concentrated in large and small vesicles in the supra- and infranuclear cytoplasm of cells in the areolae. In an ultrastructural study of the areolae, Friess et al. (1981) reported that cytoplasmic vesicles contained material of electron density similar to that of the areolar lumen and suggested that these specialized chorionic epithelial cells were responsible for absorption and intracellular transport of uterine histotroph. In the present study, heavy staining for Uf was noted in vesicles within the basal cytoplasm of cells adjacent to the chorioallantoic capillaries. In some sections there appeared to be release of Uf into capillaries. These observations suggest that Uf is transported by areolae from its site of secretion directly into the fetal circulation. This direction of Uf transport is consistent with work by Ducsay et al. (1982) which demonstrated transport of ^{59}Fe-Uf from uterine secretions into placental tissue, preferentially in areolae, followed by accumulation in fetal blood. The chorioallantoic capillaries are especially suited for macromolecular exchange by virtue of the numerous fenestrae within the capillary endothelium (Friess et al., 1981). The mechanism of macromolecular transport across epithelial membranes of the gut (Rodewald, 1973; Smith et al., 1979) and yolk sac (Linden and Roth, 1978; Moxon and Wild, 1976) of several species has been investigated and the observations may lend understanding to the Uf uptake and transport mechanism of the areolae. Two types of transport processes were described for the tissues examined. In epithelia of rabbit and chick yolk sac and

rat intestine (Linden and Roth, 1978; Moxon and Wild, 1976; Rodewald, 1973), IgG is absorbed by receptor mediated pinocytosis and transported across the cell within coated vesicles. By contrast, in the newborn pig, IgG and colostral proteins are absorbed into the intestinal epithelium by nonspecific (fluid phase) protein transport which is stimulated by high concentrations of protein in the intestinal lumen and involves uptake of substances into large cytoplasmic vacuoles (Smith et al., 1979). These vacuoles are similar to those observed within cells of the areolae in the present study suggesting nonspecific protein transport by these structures.

Chen et al. (1975) suggested that Uf transport from the areolae occurred through the chorioallantoic mesoderm and allantoic epithelium and into the allantoic sac, implying movement across several cell layers. They observed faint fluorescent staining for Uf within the cells of the chorioallantoic mesoderm; however, comparable results were not obtained in the present study. Although the reason for this discrepancy is not known, it may be related to technique differences, because unlabeled antibody techniques such as the PAP method are more sensitive and specific than methods employing a detector molecule covalently bound to the antibody as in immunofluorescence (Sternberger, 1969). Thus, the chorioallantoic mesodermal fluorescence (previously reported) may have been nonspecific.

Palludan et al. (1969) administered ^{55}Fe to pregnant gilts and examined the distribution of Fe within placental tissue by autoradiography. A high density of grains representing ^{55}Fe were observed in the areolae and uterine glandular epithelium and secretions. Since maternal-to-fetal Fe transport is probably mediated by Uf (Ducsay et al., 1982), the data of Palludan et al. (1969) support the role of areolae in transport of Uf.

In addition, Palludan et al. (1969) observed few ^{55}Fe grains in stroma surrounding the areolae which indicated that transport of iron directly across the chorioallantoic membrane into the allantoic sac was not likely.

Uteroferrin had not been measured in fetal blood prior to this study. The observed umbilical venous-arterial Uf concentration difference suggested that (1) Uf entered the fetal circulation within the placenta, thus supporting the immunohistochemical observations of release from the areolae into chorioallantoic capillaries, and (2) some of the Uf was removed from circulation by the fetus. It was calculated that approximately 36.0 ng of Uf were removed from each ml of blood during each pass through the fetus at Day 75 of pregnancy. Comline et al. (1979) estimated mean umbilical blood flow to be 200 ml/min/kg fetus during late pregnancy in pigs. Utilizing that umbilical blood flow value and the Day 75 fetal pig body weight of 0.322 kg reported by Ducsay (1980), Uf transfer to the fetus was calculated to be about 3.34 mg/day. Since Uf contains 0.167% Fe by weight (Buhi et al., 1982b), Fe transfer by Uf to the Day 75 fetus was calculated to be about 5.58 μg/day. Ducsay (1980) reported that total fetoplacental unit Fe increased 17.3 mg between Days 75 and 112 of gestation. This increase would require an average Fe uptake of 467 μg/day or 279 mg of Uf. Although the calculated average daily uptake of Uf from the umbilical circulation by the fetus is not adequate to account for fetoplacental unit requirements it should be emphasized that this figure was a single point estimate calculated with a mean value for Uf uptake. Fetal uptake of Uf was correlated (r=.993) with the amount of Uf present in the uterine vein. Variation in fetal Uf uptake and Uf transport to the umbilical vein among fetuses may indicate that there is considerable

variation in these measurements within a single fetus over time. In addition umbilical blood flow in midterm fetuses is highly variable (Carter, 1975). Time related, e.g. hourly, changes in these factors may allow adequate uptake of Uf from the umbilical venous blood to meet fetal Fe requirements. Chronic studies designed to investigate this concept are needed. In addition, there may be direct transfer of Fe from Uf to transferrin within the placenta itself, which would increase total Fe transport by the placental circulation. An alternative mode of Fe transport during late pregnancy cannot be ruled out since the rate of Uf synthesis is known to decline markedly after Day 75.

In this study, Uf was bound by fetal liver membranes suggesting that the liver is a site of Uf uptake. In contrast, Uf was not detected in liver tissue by immunohistochemical staining. Inability to detect Uf by immunocytochemistry may be due to molecular changes in Uf resulting from receptor binding or rapid metabolism of Uf and its Fe after uptake. Hematopoiesis accounts for most of the Fe requirement of the developing mammalian fetus, and the liver is the primary site of hematopoiesis throughout most of fetal life (reviewed by Pearson, 1973). Previously, Bazer et al. (1975) demonstrated accumulation of Uf in pig allantoic fluid. Loss of Fe by Uf to allantoic transferrin (Buhi et al., 1982a) and subsequent uptake of transferrin from the allantois (Ducsay et al., 1982) is followed by loss of transferrin Fe at the liver, indicating an indirect role of Uf in Fe transport. However, uptake of circulating Uf by the fetus and binding of Uf by liver membranes, as demonstrated in this study, emphasize a more direct role of Uf in Fe transport to the primary site of hematopoiesis. This mechanism is supported by the work of Ducsay (1980) who administered ^{59}Fe intravenously to pregnant gilts and

found high levels of radioactivity in fetal tissues (including liver) 24 h later, but little evidence for major accumulation of ^{59}Fe in allantoic fluid. This suggested that Uf accumulation in the allantois is probably secondary to its uptake by the liver and may represent a pool of "spillover" material deposited during times of excess Uf.

Detection of activatable acid phosphatase activity and results of immunodiffusion and NEPHGE analysis demonstrated that Uf was present in fetal urine which is indicative of its clearance by the kidney. These results are in agreement with those of Suarez et al. (1968) who reported an electrophoretic pattern of pig urine proteins similar to that for plasma with several large molecular weight proteins being present, including albumin (65,000 daltons). The bladder of the pig is connected, by the urachus, to the allantois during most of fetal life, and urine flow occurs at a high rate from about Day 45 onward (Patten, 1948; Perry and Stainier, 1962). Results of the present study suggested that Uf is cleared from fetal blood by the kidney and enters the allantoic sac in fetal urine.

Immunohistochemical localization of Uf within collecting ducts and proximal tubules of the fetal kidney supports the described route of Uf transport to the allantois. Inability to detect Uf within the distal tubules and tubules of the Loop of Henle is probably a result of the absorptive activity of the proximal tubules in reducing the concentration in the ultrafiltrate to levels below the sensitivity of the PAP procedure. This is supported by the observation that some cells of the proximal tubule epithelium contained positive staining for Uf. Proteins absorbed by the proximal tubules are degraded, and breakdown products are released into the peritubular space to enter surrounding capillaries

(Guyton, 1976). This mechanism may act to recover Uf bound Fe for transport to transferrin within the circulation or in Fe storage since kidney tissue contains measurable ferritin (Linder et al., 1975). Detection of Uf within collecting tubules probably reflects reconcentration of the remaining Uf by removal of water from the urine.

Results from the present study support the following model for Uf transport to the developing fetus (Fig. 4.1). Uteroferrin within secretions of endometrial uterine glands is taken up by nonreceptor mediated (fluid phase) pinocytosis into cells of the chorionic areolae, transported to the base of the areolar cells and released into the chorioallantoic capillaries. Uteroferrin is then transported by the umbilical vein directly to the fetal liver. Much of the circulating Uf is probably bound by the liver and its Fe removed for hematopoiesis and other functions. Uteroferrin not bound by the liver is partially cleared from the blood by the kidney and subsequently enters the allantoic sac along with fetal urine through the urachus. This mechanism accomplishes two purposes, i.e., direct and efficient Fe transport to the site of hematopoiesis and storage of Fe in the allantoic fluid to meet subsequent requirements. It is possible that other mechanisms for iron transport from dam to conceptus exist; however, they have not yet been demonstrated.

CHAPTER V
UTEROFERRIN BINDING BY THE FETAL LIVER: CELLULAR SPECIFICITY AND MECHANISM OF UPTAKE

Introduction

Uteroferrin (Uf) is an iron (Fe) containing glycoprotein secreted by the porcine uterus to transport Fe to the developing conceptus (Ducsay et al., 1982). Experiments described in Chapter IV demonstrated that Uf was present in fetal blood and that a significant umbilical venous-arterial difference in Uf concentration was detected on Day 75 of gestation which indicated fetal removal of Uf from umbilical vein blood. In addition, Uf was specifically bound by liver membranes prepared from Day 75 fetuses which suggested that liver removed a portion of circulating Uf during passage through the fetus (Chapter IV). Liver is a major site of hematopoiesis in the developing pig fetus (Du Bois, 1963), and uptake of circulating Uf probably functions to deliver Fe for hemoglobin production. The mechanism for removal of Fe from Uf following binding to the liver is not known but may require endocytosis of bound Uf and subsequent degradation of the protein molecule. Recently, Willingham and Pastan (1980) described a mechanism for endocytosis of $\alpha 2$ macroglobulin bound to specific cell surface receptors in cultured fibroblasts. They suggested that binding of ligand to a specific surface receptor induces uptake, by endocytosis, at sites on the plasma membrane known as coated pits. These specialized regions are identified by their invaginated shape and the presence of a fuzzy layer (clathrin) on the cytoplasmic

side of the plasma membrane. Following endocytosis, ligand and receptor or ligand only are transferred to another structure known as a receptosome (Willingham and Pastan, 1980). Receptosomes do not contain hydrolytic enzymes but move by saltatory motion to portions of the cytoplasm which contain lysosomes. There, endocytosed molecules enter lysosomes and are degraded (Willingham and Pastan, 1980). This mechanism of receptor-mediated endocytosis has been suggested for uptake of other receptor bound macromolecules in various tissues (Willingham et al., 1981; Ashwell and Harford, 1982) since coated pits are involved in endocytosis of most receptor bound molecules examined (Goldstein et al., 1979; Wall et al., 1980). Receptor-mediated uptake of glycoproteins by liver tissue in vivo and in vitro has been extensively examined (reviewed by Ashwell and Harford, 1982) and three distinct plasma membrane receptor systems were described for mammalian liver tissue. These include (1) a specific hepatocyte receptor for asialoglycoproteins terminating in a nonreducing galactose residue; (2) a specific receptor (located on endothelial and Kupffer cells lining the liver sinusoids) for binding glycoproteins with terminal mannose or N-acetylglucosamine residues; and (3) a specific hepatocyte receptor for glycoproteins bearing fucose residues bound in $\alpha 1 \rightarrow 3$ linkage to N-acetylglucosamine. Localization of ^{125}I-labeled glycoproteins in rat liver by electron microscope autoradiography (EM-ARG) (Hubbard and Stukenbrok, 1979; Hubbard et al., 1979) suggested that the mechanism for endocytosis of ligands bound to receptor systems (1) and (2) is similar to that described by Willingham and Pastan (1980). The present study was designed to determine by ARG the specific cell type of the fetal liver (Kupffer, endothelial or hepatocyte) that binds Uf and to examine the mechanism for uptake of hepatic receptor bound Uf.

Materials and Methods

Animals and Surgical Procedures

Estrus detection, breeding and surgical procedures were performed as described in Chapter III.

Iodination of Purified Uteroferrin

Uteroferrin was purified by the method of Buhi et al. (1982a). Purified Uf was iodinated by the Iodo-Gen technique (Markwell, 1978; Markwell and Fox, 1978) using a modification of the procedure of Buhi et al. (1982a). Acid washed 13 x 100 mm tubes were coated with 100 μg of Iodo-Gen (1,3,4,6-tetrachloro 3α,6α-diphenylglycoluril; Pierce Chemical Company, Rockford, IL) by evaporation of chloroform. Uteroferrin (135 μg) in 1 ml of iodination buffer (0.075 M sodium barbital, 0.4 M NaCl, pH 8.0) was added to the Iodo-Gen tube previously rinsed in buffer. Carrier-free Na ^{125}I (3.0 mCi) was added and the tube shaken a few seconds every minute for a total of 15 min. The ^{125}I-Uf was separated from unreacted Na ^{125}I by dialysis overnight against two changes of phosphate buffered saline (Dulbecco) at 4°C. Specific activity of the prepared sample was 2.8 mCi/mg protein.

Infusion of ^{125}I-Uteroferrin

Uteroferrin binding to fetal liver was examined by ARG following infusion of ^{125}I-Uf into the umbilical vein of fetuses on Day 75 of gestation. Two gilts were laparotomized on Day 75 and the uterus exposed to allow exteriorization of each fetus through an incision in the adjacent antimesometrial wall of the uterus, chorioallantois and amnion.

^{125}I-uteroferrin (135 x 10^8 cpm, ~35 µg Uf) in 0.250 ml of buffer was injected into the umbilical vein of treated fetuses (n=3), using a 1 cc tuberculin syringe fitted with a 22 gauge needle. At 3 min after infusion of ^{125}I-Uf, the umbilical arteries and vein were tied with silk suture in two adjacent areas approximately 5 cm apart and the fetus removed by severing the umbilical cord at a point between the ties. Immediately upon removal of the fetus, the umbilical vein was catheterized with polyvinyl tubing and the ties removed. The fetus was perfused first with normal saline until there was a clear fluid effluent from the umbilical arteries (~1 min) and then with fixative (2% w/v paraformaldehyde, 0.1% w/v glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.0) for 2 to 3 min. To examine the specificity of ^{125}I-Uf binding, excess unlabeled Uf (3.5 mg) was injected into the umbilical vein of one fetus 1 min prior to injection of ^{125}I-Uf. Other treated fetuses received an equal volume of 0.15 M NaCl in place of unlabeled Uf.

Preparation of Fetal Tissues for Light and Electron Microscopy

After perfusion fixation, liver and heart tissue (used as a tissue specificity control) were cut into 1-3 mm^3 pieces and fixation continued overnight at 4°C by immersion in fixative. Tissues were postfixed, dehydrated and embedded as described in Chapter III for electron microscopic ultrastructure. Thin sections (80-90 nm) and thick sections (500 nm) were cut on a LKB Ultratome III ultramicrotome.

Light Microscope Autoradiography

Thick sections were mounted on gelatin chrome alum coated glass slides and prepared for light microscope autoradiography (LM-ARG) by

dipping in a 1:1 aqueous dilution of Ilford K·5 emulsion (30°C). After an exposure time of 3 to 5 weeks, slides were developed for 2 min in Dektol (1:1 dilution; Eastman Kodak, Rochester, NY) at 23°C, placed in stop bath (1% (v/v) glacial acetic acid) for 20 to 30 min, and fixed in 10% sodium thiosulfate for 4 to 8 min. Developed sections were stained with Richardson's stain (Richardson et al., 1960) for 4 min at 50°C, rinsed in 95% (v/v) ethanol and stained with toluidine blue (1% (w/v) in 0.15 M phosphate buffer at pH 7.0) for 2 min at 50°C. Sections were observed and photographed on a Wild M-20 optical microscope.

Electron Microscope Autoradiography

Thin sections were collected on formvar coated nickel grids and the loop technique of Williams (1977) was followed for electron microscope autoradiography (ER-ARG) using Ilford L-4 emulsion. Several blank grids were included for determination of background. After exposure for 5 to 10 weeks at 0°C, the grids were developed by a modification of the technique of Stevens (1966). Briefly, solutions were freshly prepared and filtered (0.45 μ, Millipore Corp., Bedford, MA) prior to use, and all steps through fixation were performed by illumination from a safelight (15 watt bulb, Wratten OA filter, at least 3 m from work area). Exposed grids were initially immersed in 100% ethanol for 3.5 min at 25°C and then dried on filter paper. Grids were developed by flotation on a drop of Microdol-X (Eastman Kodak, Rochester, NY) for 6 min at 23°C, and development was terminated by rinsing grids for 5 min in a spot plate containing distilled water. Grids were fixed by immersion for 10 min in a solution containing 1.5 M $Na_2S_2O_3$, 0.13 M $Na_2S_2O_5$ and 0.08 M Na_2SO_3, rinsed as previously described, washed with a stream of distilled water and dried on filter

paper. Preliminary experiments indicated that removal of gelatin in the emulsion, that remained after photographic processing, was necessary to obtain acceptable micrographs. To remove gelatin, grids were immersed in distilled water for 30 min at 37°C, floated on a solution of 0.5 N acetic acid for 15 min at 37°C, rinsed with a stream of distilled water and finally soaked in distilled water for 10 min at room temperature. Grids were poststained in three changes (20 min each) of saturated aqueous uranyl acetate at 50°C followed by lead citrate (Reynolds, 1963) for 30 min at 50°C. Sections were examined and photographed on a JEOL 100-CX transmission electron microscope operated at 80 KV.

Results

Light Microscope Autoradiography

Fetal liver localization of ^{125}I-Uf by LM-ARG was utilized to allow examination of larger areas of fetal tissue than are possible by electron microscopy. Specificity of ^{125}I-Uf localization in fetal liver, by competitive inhibition with excess Uf and by examination of fetal heart tissue, was determined only by LM-ARG.

A few ARG grains were randomly distributed over all slides examined in areas with and without tissue (Fig. 5.1) which indicated that these grains represented background exposures not associated with the presence of ^{125}I-Uf. Heavy accumulations of grains representing ^{125}I-Uf were associated with cells lining the liver sinusoids following infusion of this molecule into the fetal umbilical vein (Fig. 5.2). However, it was not possible to determine the specific cell type(s), i.e., Kupffer versus endothelial, which accumulated ^{125}I-Uf. Specific accumulations of ARG

Figure 5.1. Light microscope autoradiograph of Day 75 fetal liver fixed by perfusion 3 min after injection of ^{125}I-Uf into the umbilical vein. Note grains are concentrated over Kupffer and/or endothelial (KE) cells lining liver sinusoids (S). Grains within the tissue not associated with KE cells are distributed in a pattern similar to areas of the slide not containing tissue (*). 3 wk exposure. X2200.

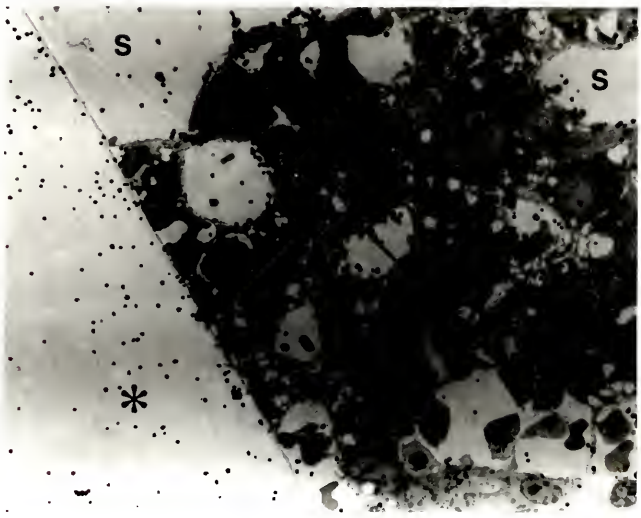
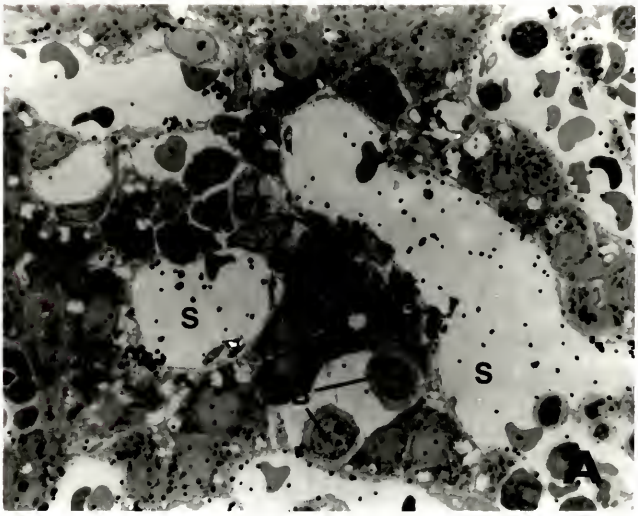
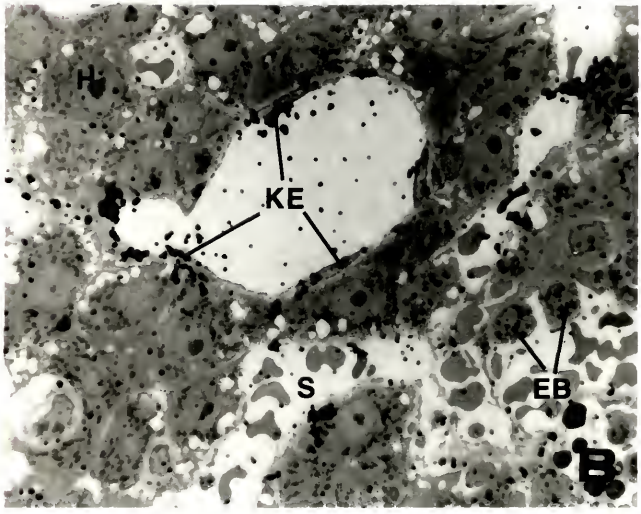
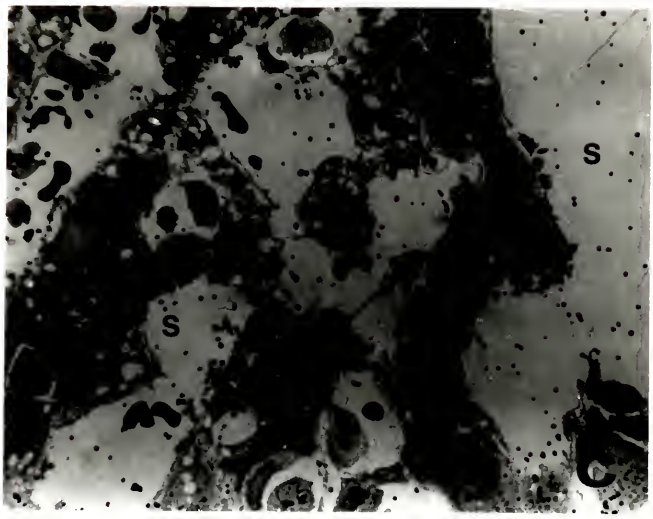

S
S
*

Figure 5.2. Light microscope autoradiographs of Day 75 fetal liver fixed by perfusion 3 min after injection of ^{125}I-Uf into the umbilical vein. Grains are concentrated in Kupffer and/or endothelial cells (KE) lining the liver sinusoids but not in hepatocytes (H) or erythroblasts (EB). Three examples from different blocks of tissue are shown in (A), (B) and (C). 3 wk exposure. X2200.


S
S
A


H
KE
S
EB
B


S
S

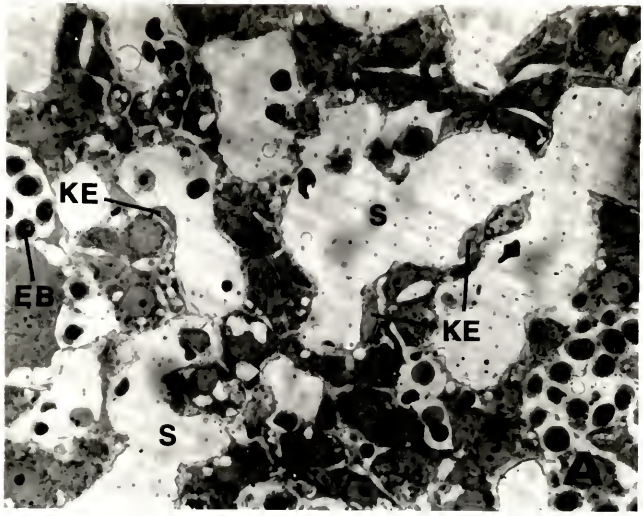
grains above background levels were not associated with hepatocytes or erythroblastic tissue (Fig. 5.2). Injection of excess unlabeled Uf into the umbilical vein 1 min prior to injection of ^{125}I-Uf eliminated accumulation of ARG grains in cells of the liver sinusoids (Fig. 5.3) but a change in the distribution of grains associated with hepatocytes and erythroblasts was not observed (Fig. 5.3). Grains representing ^{125}I-Uf were not observed in heart tissue from fetuses receiving ^{125}I-Uf alone (Fig. 5.4a) or after prior injection of excess unlabeled Uf (Fig. 5.4b).

Electron Microscope Autoradiography

Localization of ^{125}I-Uf by EM-ARG was performed to examine the mechanism for uptake of Uf bound by fetal liver tissue. Heart tissue or liver tissue from animals given excess unlabeled Uf prior to an injection of ^{125}I-Uf were not examined by EM-ARG because specificity of ^{125}I-Uf localization was determined by LM-ARG and evidence for uptake of Uf could not be obtained from these tissues due to the lack of specific localization of ^{125}I-Uf.

Exposed grains representing ^{125}I-Uf were present almost exclusively within cells lining the liver sinusoids (Fig. 5.5) although a few grains were associated with hepatocytes (Fig. 5.5b, 5.6f and 5.7) and erythroblasts (not shown). It was not possible to distinguish Kupffer and endothelial cells by morphological examination in this study; therefore, the distribution of ARG grains between these cell types was not determined. Positive identification would require cytochemical demonstration of endogenous peroxidase activity which is present in Kupffer, but not endothelial cells. Within Kupffer and endothelial cells, grains were frequently associated with coated pits, coated vesicles, and smooth

Figure 5.3. Light microscope autoradiographs of Day 75 fetal liver fixed by perfusion 3 min after injection of ^{125}I-Uf into the umbilical vein. Excess unlabeled Uf was injected 1 min prior to injection of ^{125}I-Uf. Grains are not concentrated in Kupffer and/or endothelial cells, hepatocytes or erythroblasts (compare with Fig. 5.2). Two examples from different tissue blocks are shown in (A) and (B). 5 wk exposure. X1450.


KE
EB
S
KE
S
A

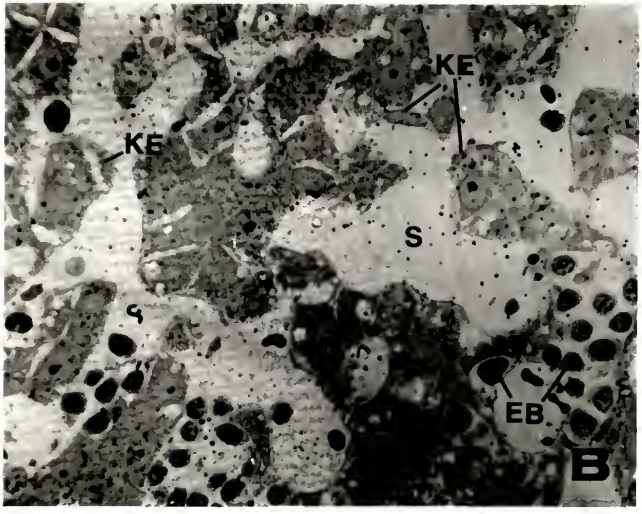
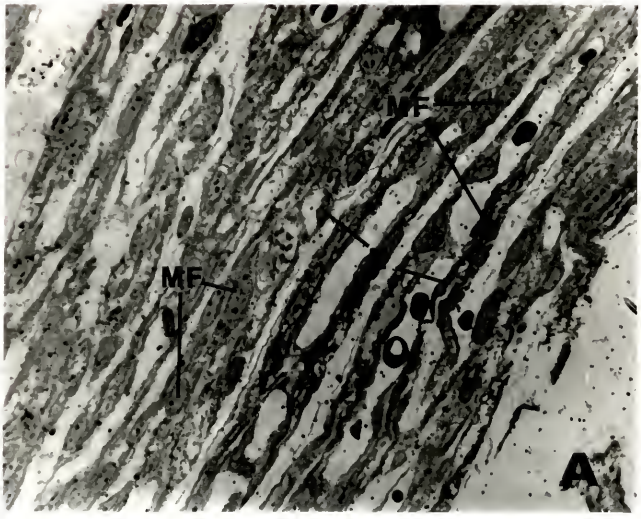

KE
KE
S
EB
B

Figure 5.4. Light microscope autoradiographs of Day 75 fetal heart fixed by perfusion 3 min after injection of ^{125}I-Uf into the umbilical vein. Grains are not concentrated in muscle fibers (ME) or capillary endothelial cells (E) of heart tissue from fetuses receiving (A) 0.15 M NaCl or (B) excess Uf prior to injection of ^{125}I-Uf. 5 wk exposure. X1450.


MF
MF
A

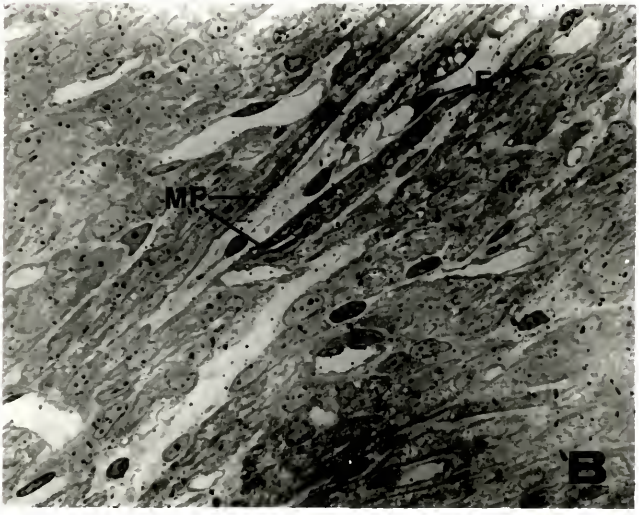
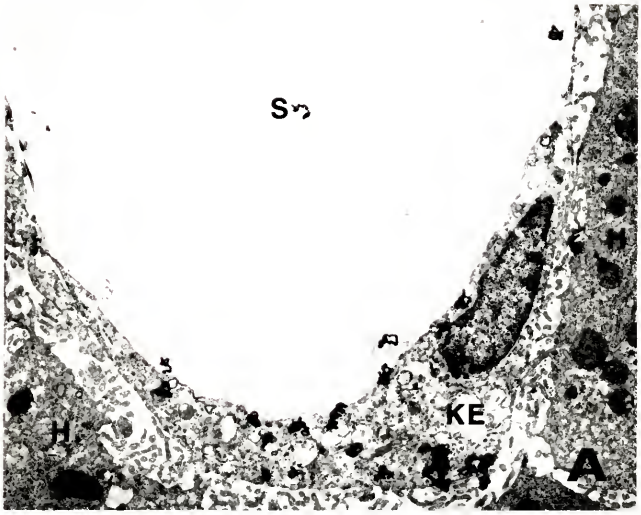

MP
B

Figure 5.5. Electron microscope autoradiographs of Day 75 fetal liver fixed by perfusion 3 min after injection of ^{125}I-Uf into the umbilical vein. Exposed grains are concentrated in Kupffer and/or endothelial (KE) cells that line liver sinusoids. Few grains are present in hepatocytes. Two examples from different areas of liver tissue are shown in (A) and (B). 10 wk exposure. X8500.


S
KE
H
A

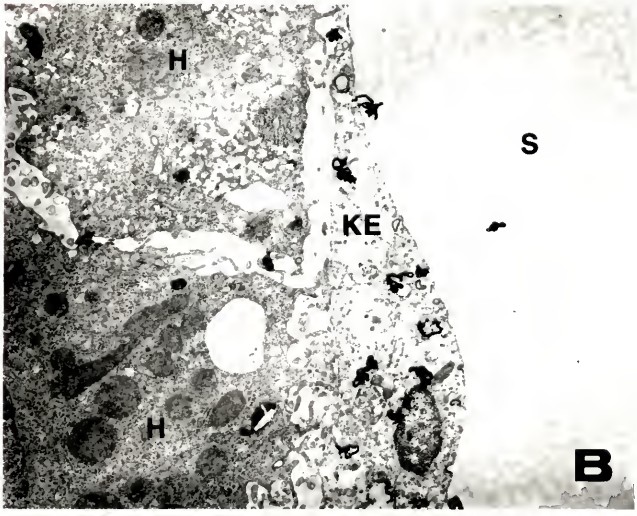
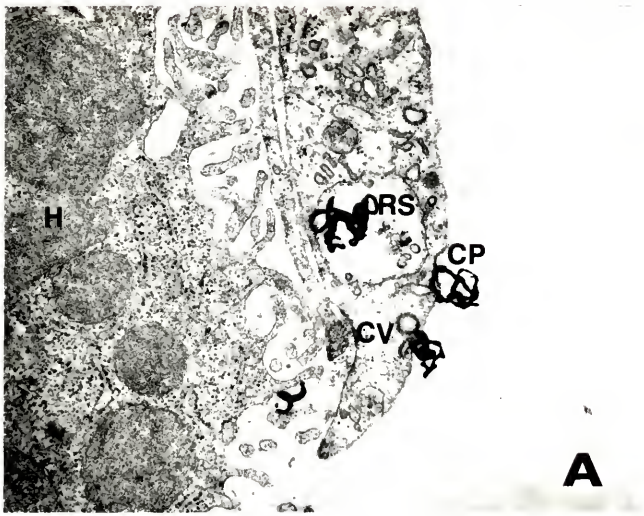
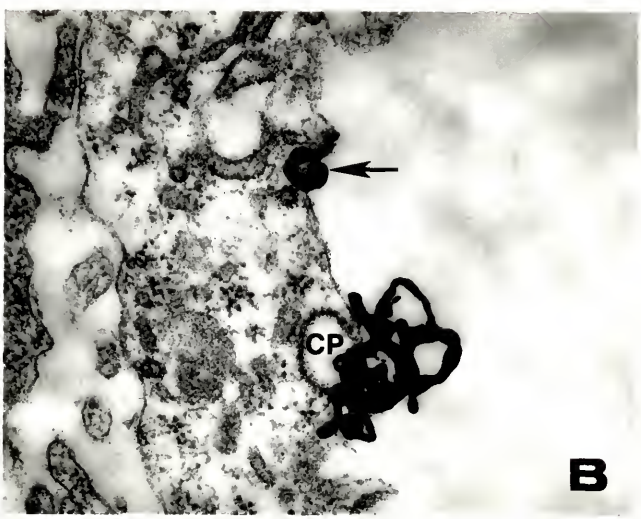
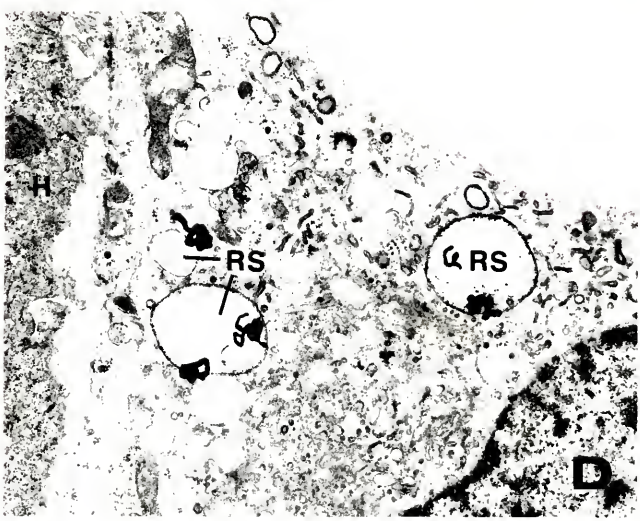

H
S
KE
H
B

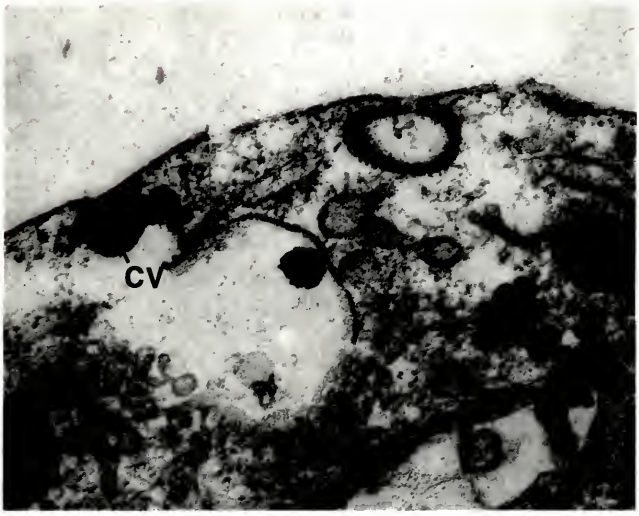
Figure 5.6. Electron microscope autoradiographs of Day 75 fetal liver fixed by perfusion 3 min after injection of ^{125}I-Uf into the umbilical vein. A) Exposed grains in a KE cell are associated with coated pits (CP), coated vesicles (CV) and smooth membrane vesicles that resemble receptosomes (RS). 10 wk exposure. X26,000. B) Exposed grains are associated with an invaginated coated pit and an unidentified area (arrow) of the plasma membrane of a KE cell. 10 wk exposure. X65,000. C) KE cell with ARG-grain present in smooth membrane receptosome-like vesicle. Note close association of receptosome to coated vesicle. 5 wk exposure. Nucleus (N). X43,000. D) Exposed grains are associated with receptosome-like vesicles in the cytoplasm of a KE cell. 5 wk exposure. X16,900. E) KE cell with exposed grains present in a coated pit and other unidentified areas of the cytoplasm (arrows). 5 wk exposure. X65,000. F) Exposed grains in KE cell and hepatocyte. Note that some grains are associated with coated pits and coated vesicles of the KE cell. Other grains in KE cell and hepatocyte are not associated with a recognizable cytoplasmic structure (arrows). 10 wk exposure. X33,800.


H
ORS
CP
CV
A


CP
B

C




CV

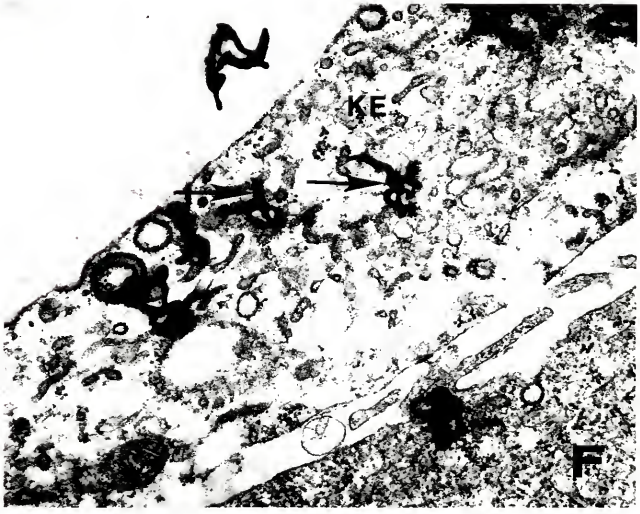
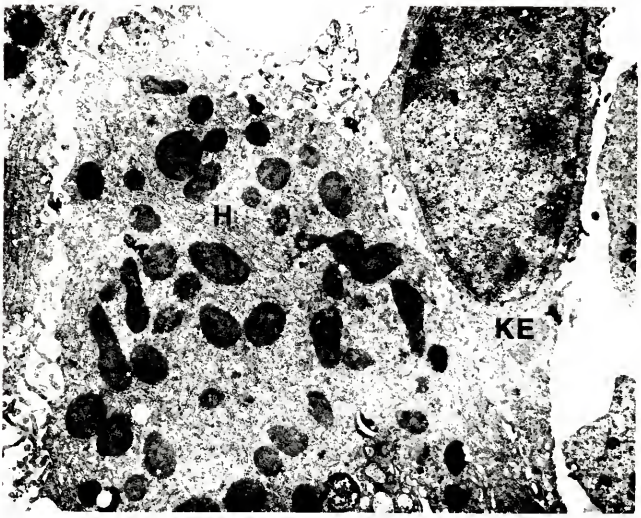

KE
F

Figure 5.7. Electron microscope autoradiograph of Day 75 fetal liver fixed by perfusion 3 min after injection of ^{125}I-Uf into the umbilical vein. Exposed grains present in a hepatocyte are not associated with a specific cytoplasmic structure. 10 wk exposure. X8500.


H
KE

membrane vesicles similar to "receptosomes" described by Willingham and Pastan (1980) (Fig. 5.6). The low resolution (1000 Å) of ^{125}I-ARG and the small size of many of the vesicles observed (coated vesicles ≃ 1200 Å) prevented definitive assessment of the association of grains with these structures. However, the frequency of grains observed to be located directly over these structures suggested that they contain endocytosed ^{125}I-Uf. Occasionally, in Kupffer and endothelial cells, grains were observed not associated with a particular type of cytoplasmic organelle (Fig. 5.6e). Similarly, ARG grains present in hepatocytes (Fig. 5.6f and 5.7) and erythroblasts were not localized in a particular cytoplasmic structure.

Discussion

Specific uptake of a wide variety of molecules by cells involves receptor-mediated endocytosis (reviewed by Goldstein et al., 1979). Systems which have been extensively investigated include endocytosis of low density lipoproteins by fibroblasts, smooth muscle cells and endothelial cells, uptake of yolk proteins by chicken and mosquito oocytes, endocytosis of α-2-macroglobulin by fibroblast, macrophages and 3T3 cells, and endocytosis of asialoglycoproteins by hepatocytes. Endocytosis by these systems involves binding to specific cell surface receptors and subsequent aggregation of the receptor-ligand complex into specialized regions of the plasma membrane termed coated pits (Roth and Porter, (1964). Previous studies (reviewed by Goldstein et al., 1979) suggested that receptor-bound ligand was internalized by invagination of coated pits and subsequent formation of coated vesicles within the peripheral cytoplasm of the cell. Coated vesicles were believed to be responsible

for delivery of the ligand and/or receptor to its specific intracellular site, whether it be the lysosomes, as was the case for low density lipoproteins, α-2-macroglobulin and asialoglycoproteins or yolk granules, as was the case for yolk proteins.

Recently Willingham et al. (1981) and Willingham and Pastan (1980) examined receptor-mediated endocytosis of α-2-macroglobulin into mouse fibroblast by electron microscopy with special attention to events occurring after receptor binding. These authors determined that coated vesicle formation did not occur following coated pit invagination. Rather, they described the formation of "cryptic coated pits" which were not functionally in contact with the cell surface, as determined by exclusion of impermeant cell surface markers, but which remain physically connected to the cell surface (Willingham et al., 1981). Willingham et al. (1981) suggested that previously reported coated vesicle formation was caused by examination of plasma membranes at only one plane of sectioning. This might yield structures with the appearance of coated vesicles that are attached to the plasma membrane at another plane of the tissue. Endocytosed material was subsequently transported to smooth coated vesicles termed receptosomes (Willingham and Pastan, 1980). Receptosomes did not contain hydrolytic enzymes, but transported their contents to lysosomes for degradation. If this pathway (coated pits, cryptic coated pits and receptosomes) is common to receptor-mediated endocytosis of all molecules, a mechanism must exist to direct molecules not destined for lysosomes, e.g., yolk proteins, to the correct compartment.

Five carbohydrate-specific receptors which bind glycoproteins have been identified in mammalian and nonmammalian tissues (reviewed by Ashwell and Harford, 1982). These include (1) a receptor present on mammalian

hepatocytes that binds circulating asialoglycoproteins with terminal nonreducing galactose residues, (2) a receptor present on the plasma membrane of hepatic Kupffer and endothelial cells that binds circulating glycoproteins with terminal mannose or N-acetylglucosamine residues, (3) a receptor present on hepatocytes that binds glycoproteins with carbohydrate side chains containing fucose linked α 1→3 to N-acetylglucosamine, (4) a receptor specific for glycoproteins with terminal mannose-6-phosphate residues located on fibroblasts and hepatocytes, and (5) a receptor present on hepatocytes from chickens which binds glycoproteins with terminal N-acetylglucosamine. This last receptor type is believed to be related to the mannose/N-acetylglucosamine receptor described for Kupffer and endothelial cells of mammalian liver (Ashwell and Harford, 1982).

Within liver tissue, carbohydrate-specific receptors are believed to be involved in homeostasis of circulating glycoproteins by removal and destruction of molecules that have undergone degradation in plasma to expose the appropriate carbohydrate residue for uptake. Hubbard et al. (1979) demonstrated by EM-ARG that glycoproteins were endocytosed and transported to lysosomes within 30 min after binding to galactose and N-acetylglucosamine receptors in liver tissue.

In the present study ^{125}I-Uf was localized within Kupffer and endothelial cells which suggested that Uf may bind to fetal hepatic tissue (see Chapter IV) by mannose/N-acetylglucosamine receptors present on these cell types (Hubbard et al., 1979; Maynard and Baenziger, 1981). This is supported by the fact that large quantities of mannose, but no sialic acid residues, are present in carbohydrate chains from Uf (Chen et al., 1973). In addition, studies undertaken since this work was

concluded demonstrated that Uf binding by isolated fetal liver membranes is inhibited by ovalbumin, glycopeptides from ovalbumin, and yeast mannans, all molecules with a high mannose content (P.T.K. Saunders, personal communication). In the present study, infusion of excess Uf eliminated ^{125}I-Uf localization in Kupffer and endothelial cells, which indicated that binding was mediated by a specific saturable receptor rather than by nonspecific macro- or micropinocytosis (Willingham and Yamada, 1978; Willingham et al., 1979). Failure to localize ^{125}I-Uf in heart tissue indicated that endothelial cells of cardiac muscle capillaries, unlike endothelial cells of liver sinusoids, do not take up glycoproteins by the mannose/N-acetylglucosamine-specific receptor. This emphasizes the tissue specificity of these receptor systems.

Ultrastructural localization of ^{125}I-Uf within coated pits, coated vesicles and smooth membrane receptosome-like vesicles suggested that the mechanism of Uf endocytosis is similar to that described for α-2-macroglobulin by Willingham et al. (1981). Uteroferrin was not localized in lysosomes; however, the relatively short interval between infusion of ^{125}I-Uf and tissue fixation (≃4 min) was probably insufficient for transport of endocytosed ^{125}I-Uf to lysosomes. Willingham and Pastan (1980) reported that horseradish peroxidase labeled α-2-macroglobulin was not present in lysosomes of cultured fibroblast until 15 to 30 min after exposure of cells to the ligand. Hubbard and Stukenbrok (1979) reported a similar time (15 min) for localization of ARG grains in dense cytoplasmic bodies of hepatic sinusoidal cells after infusing ^{125}I-glycoproteins with carbohydrate chains containing terminal mannose and N-acetylglucosamine into intact rats. In addition, degradation of these

macromolecules, as determined by quantification of liver ^{125}I-monoiodotyrosine, was not detected until 15 min after ligand infusion (Hubbard and Stukenbrok, 1979).

Although Uf degradation following endocytosis was not determined in this study its ultimate breakdown by lysosomal enzymes is likely. Degradation of Uf would liberate iron (Fe) which could be utilized for hemoglobin production by erythroblastic tissue present in the fetal pig liver. Sorenson (1960) examined hematopoiesis in the liver of fetal rabbits by electron microscopy and observed transfer of ferritin-bound Fe from hepatocytes and endothelial cells to erythroblastic cells by a process previously identified and termed ropheocytosis (Policard and Bessis, 1958). This mechanism involves invagination of a ferritin containing cytoplasmic process of one cell into an adjacent cell and subsequent endocytosis of a portion of the cytoplasmic process to form a vesicle containing transferred ferritin. This mechanism may operate to direct Fe to erythroblastic tissue following degradation of Uf in the Kupffer and endothelial cells of the fetal pig liver.

Data from this study confirm previous results (Chapter IV) which indicate that Uf is bound to hepatic tissue by a specific receptor. In addition, hepatic cell types (Kupffer and endothelial) which bind Uf were identified. Ultrastructural localization ^{125}I-Uf suggested that uptake of Uf by these cells involves a previously described mechanism for cell-mediated endocytosis.

CHAPTER VI
GENERAL DISCUSSION

Maternal recognition of pregnancy in pigs occurs between Days 11 and 12 of gestation, and the presence of conceptuses in the uterine lumen at this time prevents CL regression (Dhindsa and Dzuik, 1968). Prostaglandin $F_{2\alpha}$ synthesized by the uterine endometrium (Patek and Watson, 1976) and released into the uterine venous drainage (utero-ovarian vein) is believed to be the luteolytic agent in swine (Moeljono et al., 1976). Release of $PGF_{2\alpha}$ into the utero-ovarian vein is decreased, and accumulation of $PGF_{2\alpha}$ and total protein in the uterine lumen are increased in pregnant and pseudopregnant gilts compared to nonpregnant gilts between Days 12 and 17 after the onset of estrus (Moeljono et al., 1977; Frank et al., 1977; Frank et al., 1978; Zavy et al., 1980). Chen et al. (1975) examined Uf localization by immunofluorescence in endometrial tissue obtained from gilts during the estrous cycle and early pregnancy. They observed Uf in epithelial cells and lumina of uterine glands on Days 9 and 12 of the estrous cycle and early pregnancy which suggested synthesis of Uf by the glandular epithelium and release of Uf toward the uterine lumen (exocrine release). This interpretation was supported by subsequent in vitro data which indicated synthesis of Uf by endometrial tissue taken from gilts during diestrus and early pregnancy (Basha et al., 1979; Roberts et al., 1982). On Days 15 and 18 of the estrous cycle, Chen et al., (1975) observed that fluorescence was decreased in epithelial cells and lumina of uterine glands, but intense in stroma surrounding the

glands. These data were interpreted to indicate a change in the direction of release of Uf toward the endometrial stroma where it could enter the vasculature (endocrine release). In pregnant animals, Uf release appeared to continue in an exocrine fashion.

Based on these data, Bazer and Thatcher (1977) proposed a mechanism for maternal recognition of pregnancy in swine. These authors suggested that estradiol of blastocyst origin maintains exocrine release of endometrial secretory products, including $PGF_{2\alpha}$, and thereby preserves CL function. In nonpregnant gilts, $PGF_{2\alpha}$ would be released into the uterine vasculature between Days 12 and 15 to cause CL regression.

A portion of the research in this dissertation was designed to examine cellular mechanisms that control the shift in direction of release of uterine secretory products during the period of maternal recognition of pregnancy. No evidence to identify these mechanisms was obtained. However, results of these studies will be briefly discussed in the following paragraphs with emphasis on additional studies which may be conducted to aid in identifying cellular mechanisms involved in maternal recognition of pregnancy.

Chen et al. (1973) detected Uf by immunofluorescence antibody techniques in surface epithelial cells of the uterine endometrium during diestrus, which suggested Uf synthesis and release by these cells. In the present study Uf was not detected by the peroxidase-antiperoxidase technique in the surface epithelium at any time during diestrus or early pregnancy. As previously discussed (Chapter III), absence of detectable staining in the present study may be the result of tissue fixation and processing by conventional chemical methods; however, other reasons are equally plausible. The low resolution afforded by immunofluorescent

staining may have caused staining associated with uterine secretions adhered to the plasma membrane of surface epithelium to be inaccurately assessed as present within cells of the epithelium. Peroxidase-anti-peroxidase staining of Uf in uterine secretions coating the surface epithelium was frequently observed in the present study. Dantzer et al. (1981) observed release of secretory products by uterine luminal epithelium during pregnancy; however, products secreted by these cells were not identified. Wislocki and Dempsey (1946) reported considerable variation in the histochemical staining pattern (lipids, acid phosphatase, iron, alkaline phosphatase) between surface and glandular epithelium of endometrium from pregnant pigs, which indicated possible differences in secretory products synthesized by these two epithelia.

Tight junction morphology was examined by transmission electron microscopy to determine if a change in the permeability of these structures is the mechanism controlling direction of release of uterine secretory products. Claude and Goodenough (1973) reported that basal-to-apical length of epithelial occluding junctions was inversely related to the permeability of the junction, and Murphy et al. (1982) reported that the basal-to-apical length of tight junctions in uterine endometrial epithelium of rats is modified during early pregnancy. In the present study, there did not appear to be a change in the structure of tight junctions between cells of the uterine glandular epithelium during the period examined, which suggested that tight junctions are not involved in directing release of uterine secretions. Techniques which would allow better assessment of the structure of tight junctions may reveal changes not apparent by conventional transmission electron microscopy. Methods to examine the permeability of tight junctions could include freeze-fracture

analysis of plasma membrane particles associated with tight junctions and infusion of lanthanum nitrate into the pig uterine lumen. Claude and Goodenough (1973) demonstrated by freeze-fracture analysis that fewer strands of plasma membrane protein particles are associated with tight junctions from "leaky epithelia" compared with "tight epithelia."

One function of basement membranes is to restrict movement of macromolecules, and restriction is based on size with small molecules crossing basement membranes more easily than large molecules (Farquhar, 1978). Molecules of sufficient size are completely inhibited from crossing basement membranes. Bazer and Thatcher (1977) suggested that the integrity of the basement membrane may be involved in controlling the direction of release of uterine secretions. In the present study no changes in density or thickness of the basement membrane of the glandular epithelium of the uterus were detected between Days 12 and 15 which suggested that the basement membrane is not involved in controlling release of uterine secretions.

Thickness of the basement membrane beneath the glandular epithelium increased between Days 10 and 12 after onset of estrus but was not affected by pregnancy status. Increased basement membrane thickness in both pregnant and nonpregnant gilts may be associated with uterine adjustments in preparation for support of a developing conceptus. Greater thickness of basement membranes may be necessary to support the eventual increased size and luminal diameter of uterine glands in middle and late gestation (Crombie, 1972).

Degradation of collagen, the primary structural component of basement membranes may affect permeability characteristics of this structure and these changes may not be detected by visual inspection.

Endometrial CLA was determined as an indirect indicator of basement membrane integrity. Collagenase activity is inversely related to collagen content of the uterus in rats (Woessner, 1969; Ryan and Woessner, 1974) and free hydroxyproline arises from collagen breakdown. Therefore, endometrial collagen and free hydroxyproline were determined as additional evidence of collagenase activity. Changes in endometrial CLA, total collagen and free hydroxyproline were not associated with the period of maternal pregnancy recognition. It is recognized that assessment of these variables in endometrial tissues may not reflect changes occurring specifically at the basement membrane, but isolation of basement membranes for analysis was not feasible at the initiation of this study.

In the present study active CLA, total collagen and free hydroxyproline concentrations were similar in pregnant and nonpregnant gilts to Day 15 after the onset of estrus. These values probably reflected changes in the uterine morphology to support development of the blastocyst. Perry and Rowlands (1962) reported that uterine growth in pigs was rapid during the first 18 days after mating. In the present study on Day 18 in nonpregnant gilts CLA and free hydroxyproline increased compared with pregnant gilts, indicative of uterine changes associated with involution prior to return to estrus (Corner, 1921). These data indicate that interaction of the blastocyst with the uterus to maintain CL function not only insures secretory functions of the uterine endometrium but also results in maintenance of uterine growth to support the developing fetus.

Secretory processes of the glandular epithelium were examined by electron microscopy and cytochemistry to determine if the shift from exocrine to endocrine release of uterine secretory products is mediated

by a change in the direction of cellular secretion. There was no evidence to indicate that direction of cellular secretion in nonpregnant gilts was reversed during the time of maternal recognition of pregnancy. Acid phosphatase stained vesicles in the basal cytoplasm, which may contain Uf, increased on Day 15; however, visual inspection indicated that the quantity of these vesicles was not different between pregnant and nonpregnant gilts. Precise determination will require morphometric analysis of the number of these vesicles present in pregnant and nonpregnant gilts. A difference in the number of vesicles would be indicative of preferential transport of Uf to the basal plasma membrane for release into surrounding stroma; however, it is important to note that actual release of secretory products at the basal plasma membrane was not observed in the present study.

Electron microscope cytochemical and morphological observations support previous data indicating a dramatic decrease in secretory activity of uterine endometrium on Day 18 of the estrous cycle and continued release of secretory products into the lumen during pregnancy (Zavy et al., 1980).

The mechanism which controls direction of release of uterine secretory products was not identified by the methods used in this study. In addition, attempts to confirm the existence of this mechanism by peroxidase-antiperoxidase localization of Uf during early pregnancy and the estrous cycle were not successful. It is possible that immunofluorescent observations made by Chen et al. (1975) were in error due to some uncontrolled aspect of the methodology; however, this possibility was not addressed directly by attempting to repeat the work described by these authors. Also, tissue preparation techniques used in the current

study, which were different from those used by Chen et al. (1975), may have removed Uf not contained within cells, i.e. stroma. Regardless of the validity of the observations made by Chen et al. (1975), the existence of a mechanism for preferential distribution of $PGF_{2\alpha}$ is necessary. Previous measurements of $PGF_{2\alpha}$ concentrations in utero-ovarian vein blood and uterine flushings in pregnant and nonpregnant gilts attest to that fact. Bito (1972a,b) demonstrated that $PGF_{2\alpha}$ does not move freely through cell membranes and that specific mechanisms are present in many tissues for controlled movement of this compound through epithelial cell layers. The identity of these mechanisms is not known.

Decreased total protein in uterine flushings of gilts after Day 15 of the estrous cycle (Frank et al., 1977; Zavy et al., 1980) was previously suggested to occur by endocrine release of secretions from the uterus (Bazer and Thatcher, 1977). However, this decrease may reflect a combination of decreased endometrial secretory activity and protease degradation of luminal contents. In the present study secretory activity of the nonpregnant uterus, as determined by electron microscopy, was significantly depressed on Day 18. Mullins et al. (1980) reported that uterine flushings obtained from gilts during the estrous cycle and early pregnancy contained the protease plasminogen activator. The specific activity of plasminogen activator in uterine flushings increased between Days 15 and 18 of cyclic, but not pregnant gilts.

In summary, cellular mechanisms which control the exocrine to endocrine shift in release of uterine secretory products could not be detected. Rather changes in cellular function and morphology became evident in nonpregnant gilts only after CL regression. These data

emphasize the role of the conceptus in maintaining CL function and therefore a progesterone-dependent uterine environment capable of supporting conceptus development.

Development of the epitheliochorial placenta in pigs causes little or no destruction of uterine or chorionic epithelium, and final attachment of the conceptus to the uterus involves interdigitation of epithelial microvilli (Brambell, 1933). Due to this noninvasive form of placentation, the maternal and fetal blood supplies are separated by six tissue layers which act as a "barrier" to nutrient and waste exchange by diffusion between mother and fetus (Grosser, 1909; Stevens, 1975). Secretions of the uterine endometrium (histotroph) are believed to supply many nutrients for development of the conceptus (Heuser, 1927; Amoroso, 1952). These secretions are taken up by chorionic areolae and subsequently distributed to the fetus (Heuser, 1927; Chen et al., 1973; Friess et al., 1981).

Oxygen delivery to tissues of the developing fetus is achieved by diffusion across placental tissues and transport by red blood cells within the fetal circulation. The liver is the site of hematopoiesis during most of fetal life, and Fe for production of hemoglobin contained in red blood cells is supplied to the conceptus in secretions of uterine glands (Palladan et al., 1969; Ducsay et al., 1982). Ducsay et al. (1982) demonstrated that Uf, a uterine gland secretory product, is the major placental Fe transport protein in pigs. The final portion of this discussion will focus on results presented in Chapters IV and V which describe placental transport and fetal utilization of Uf.

Peroxidase-antiperoxidase localization of Uf within epithelial cells of the chorionic areolae is in agreement with observations made by Chen

et al. (1975) and further demonstrates the role of the areolae in uptake of uterine secretions. Within epithelial cells of areolae, Uf was present in large cytoplasmic vesicles similar to those responsible for macromolecular uptake by epithelium of the gut (Smith et al., 1979). In the gut this process is not stimulated by ligand-receptor interaction and is termed nonspecific (fluid phase) endocytosis.

Chen et al. (1975) localized Uf by immunofluorescence in chorioallantoic stroma adjacent to areolae of placental tissue taken from gilts during midgestation. Uteroferrin is present in high concentrations in allantoic fluid (Bazer et al., 1975) and stromal localization of Uf (Chen et al., 1975) was believed to be indicative of transport from the areolae to the allantoic sac. In the present study Uf was not detected in chorioallantoic stroma on any of the days examined (60, 75, 90 and 105), and the reason for this discrepancy is not known. However, an alternate mechanism for Uf transport to the allantoic sac was determined.

Immunohistochemical examination of placental tissues demonstrated that Uf was released into placental capillaries presumably for direct transport to the fetus. The detectable umbilical vein-artery difference in radioimmunoassayable Uf was further evidence for placental release of Uf into the fetal circulation and is the only evidence available to demonstrate direct transport of uterine secretory products endocytosed by the areolae.

Detectable differences in Uf concentration between the umbilical vein and artery also suggested that Uf was removed from blood by the fetus. The liver and kidney function in removal of macromolecules from blood and therefore their role in removing Uf from fetal circulation was examined.

Uteroferrin was localized by the peroxidase-antiperoxidase technique within the lumina of proximal tubules and collecting ducts and within cells of kidneys taken from fetuses on Day 75 of gestation. In addition, Uf was detected in fetal urine by two-dimensional polyacrylamide gel electrophoresis, Ouchterlony immunodiffusion and quantification of acid phosphatase activity. These data indicated that Uf entered fetal urine as part of the ultrafiltrate produced by the kidney. Previously Suarez et al. (1968) demonstrated that macromolecules as large as Uf are present in urine samples from adult pigs.

It was concluded that a portion of Uf which enters fetal circulation is cleared by the kidney and transported to the allantoic sac for storage. Buhi et al. (1982a) reported that Fe bound to Uf was transferred to transferrin in allantoic fluid. Transferrin is then taken up by allantoic epithelium and enters the fetal circulation (Ducsay et al., 1982). In addition to Uf two other proteins were observed in urine samples in the present study that are present in uterine flushings from gilts during early gestation (Day 11). This suggested that uterine secretory proteins in addition to Uf are transported to the allantoic sac by the described mechanism (Fig. 4.1).

Uteroferrin bound to isolated Day 75 fetal pig liver membranes in a dose-dependent manner. Also, addition of increasing quantities of unlabeled Uf to the assay competitively inhibited binding of radiolabeled Uf to the membrane preparation. These data indicated that Uf is removed from circulation by a specific saturable receptor located on the plasma membrane of fetal liver cells.

Autoradiography of fetal liver tissue following injection of ^{125}I-Uf into the umbilical vein demonstrated that Kupffer and/or endothelial

cells lining the liver sinusoids, but not hepatc ytes or erythroblasts, bind Uf. Inhibition of binding by prior injecti n of excess unlabeled Uf and lack of accumulation in cells lining capi laries in cardiac tissue demonstrated the specificity of Uf binding to Ku ffer and/or endothelial cells of the liver. Kupffer and endothelial cel s from rat liver possess receptors which bind circulating glycoproteins w th terminal mannose or N-acetylglucosamine residues on their carbohydra e chains (Hubbard et al., 1979; Maynard and Baenziger, 1981). Uteroferrin contains many mannose residues (Chen et al., 1973) which suggests that Uf binds to liver tissue by the previously described mannose/N-acetylgluc samine receptor. Recently, it was demonstrated that Uf binding by isolated etal liver membranes is inhibited by ovalbumin, glycopeptides from ovalb min and yeast mannans, all molecules with a high mannose content (P.T.K Saunders, personal communication).

Hubbard et al. (1979) examined binding of g ycoproteins with terminal mannose residues to Kupffer and endothelial cell of rat liver by EM-ARG and observed localization of grains in coated pi s, coated vesicles and larger smooth membrane vesicles. Willingham et l. (1981) proposed that receptor-mediated endocytosis of macromolecules s initiated by binding to receptors that are associated with coated pit or which rapidly migrate to become associated with coated pits. ubsequently, endocytosed molecules are transported to various intracellul r compartments. Most frequently endocytosed molecules are transported to lysosomes where they are degraded. In the present study ARG grains r presenting Uf were associated with coated pits, coated vesicles anc larger smooth membrane vesicles in Kupffer and/or endothelial cells, wh ch suggested that Uf is endocytosed by the receptor mediated mechanism c scribed by Willingham

et al. (1981). Uteroferrin was not observed in lysosomes, probably due to the short interval between Uf infusion and tissue fixation (4 min). Additional studies are needed to confirm degradation by lysosomes; however, it is likely that lysosomal degradation occurs to free Fe for use in erythropoiesis. Transport of Uf bound Fe to erythropoietic tissue could be examined by injecting ^{59}Fe-Uf into the fetus as described in the current study and observing, by EM-ARG, the location of ^{59}Fe before, during and after release from Uf.

These data describe a mechanism for Uf placental transport and utilization by the fetus (Fig. 4.1). Uf secreted by uterine glands is endocytosed by epithelial cells of placental areolae. Subsequently, Uf is released into the placental vasculature and carried into the fetus via the umbilical vein. The liver binds a portion of circulating Uf, probably by a mannose specific receptor located on Kupffer and/or endothelial cells and removes the Fe bound to Uf for subsequent use in hemoglobin synthesis. Uteroferrin not bound by the liver is cleared by the kidney and transported to the allantoic sac. Within the allantoic fluid, Uf can give up its Fe to transferrin which can be absorbed into the fetal circulation to provide Fe for hematopoiesis and to meet other metabolic needs for Fe.

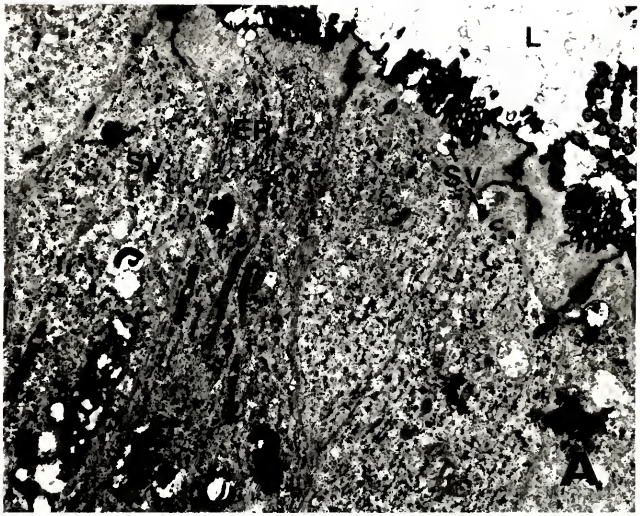
It was not possible to account for daily fetal Fe requirements (Ducsay, 1980) by the calculated (36 ng) Uf transfer; however, this value was determined from acute measurements of umbilical blood Uf concentrations. Chronic experiments designed to account for variations in Uf transfer and storage over time are needed. It is possible that other mechanisms of Uf transport to the fetus are present and that several mechanisms may allow transfer of adequate Fe to meet requirements of the developing conceptus.

In summary, a mechanism of Uf transport has been described. This mechanism accomplishes direct and efficient Fe transport to the liver which is the primary site of hematopoiesis in the fetus during most of gestation, and temporary storage of Fe in allantoic fluid to meet subsequent Fe requirements.

APPENDIX

Transmission electron micrographs of acid phosphatase activity in glandular epithelium from the porcine uterus on Days 12 and 15 postestrus.

Transmission electron micrographs of acid phosphatase activity in uterine glandular epithelium on Day 12 after onset of estrus. A) Intense acid phosphatase staining associated with the Golgi apparatus, cytoplasmic vesicles, endoplasmic reticulum, microvilli and luminal contents of secretory cells of glandular epithelium from a pregnant uterus. X9240. B) Acid phosphatase activity associated with nuclei only of secretory cells from a pregnant uterus. Note the lack of staining in the Golgi apparatus, cytoplasmic vesicles and endoplasmic reticulum. X9240. C) Acid phosphatase staining associated with apical border of secretory cells of glandular epithelium from a nonpregnant uterus. Note the variation in number of apical secretory vesicles between cells of the epithelium. X18,200. D) Acid phosphatase activity associated with glandular epithelium from a pregnant uterus. Note that staining of cytoplasmic organelles is variable. Also, staining is present in the connective tissue (CT) beneath the basement membrane. Note the variability in structure of the endoplasmic reticulum with swollen (*) and nonswollen profiles present. X2800.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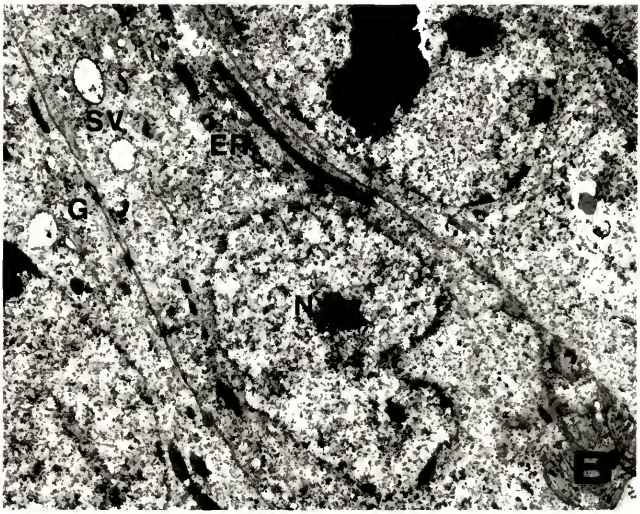
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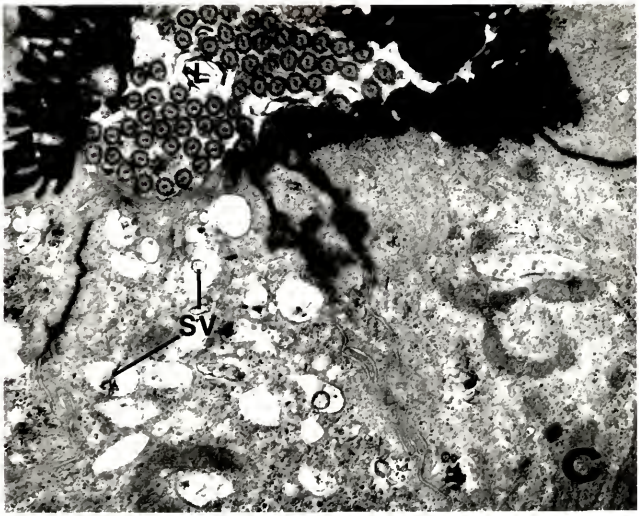
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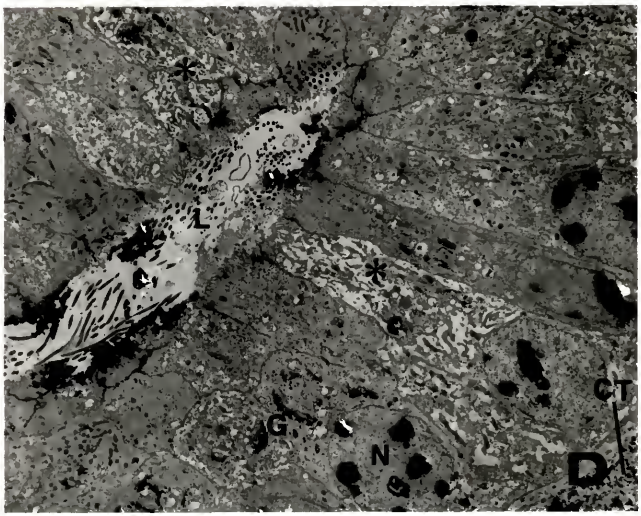
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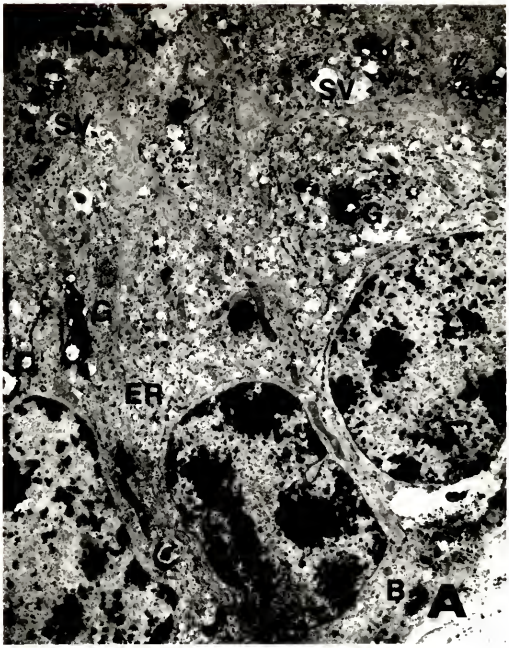
Transmission electron micrographs of acid phosphatase activity in uterine glandular epithelium on Day 15 postestrus. A) Intense acid phosphatase activity associated with microvilli, secretory vesicles, endoplasmic reticulum, the Golgi apparatus, nuclei and occasional basal vesicles (B) in secretory cells of glandular epithelium from a nonpregnant uterus. X7000. B) Acid phosphatase activity in glandular epithelium from a pregnant uterus. Note the variation in staining of the endoplasmic reticulum among secretory cells. Frequent basal vesicles with acid phosphatase staining are present. X14,900. C) Acid phosphatase staining associated with glandular epithelium from a pregnant uterus. No staining is present in nuclei or endoplasmic reticulum and very little staining is associated with the Golgi apparatus, secretory vesicles and microvilli. X7000. D) Acid phosphatase staining of glandular epithelium from a nonpregnant uterus. Note the variation in staining of the Golgi apparatus and nuclei and lack of staining in the endoplasmic reticulum. Staining for acid phosphatase is present in the intercellular space between some secretory cells. This type of staining was observed in only one or two glands. X4600.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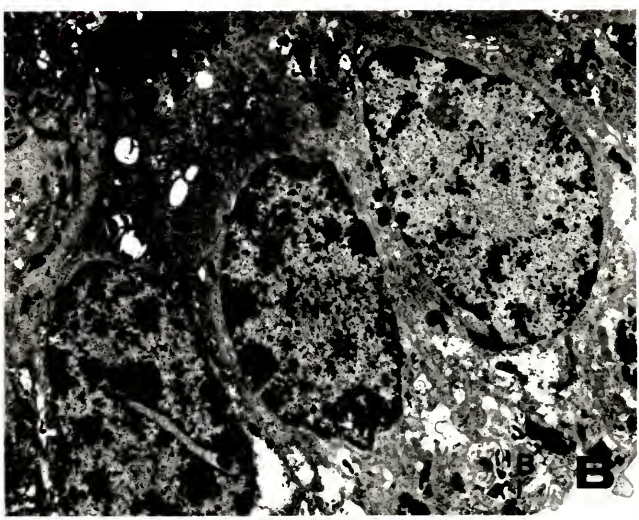
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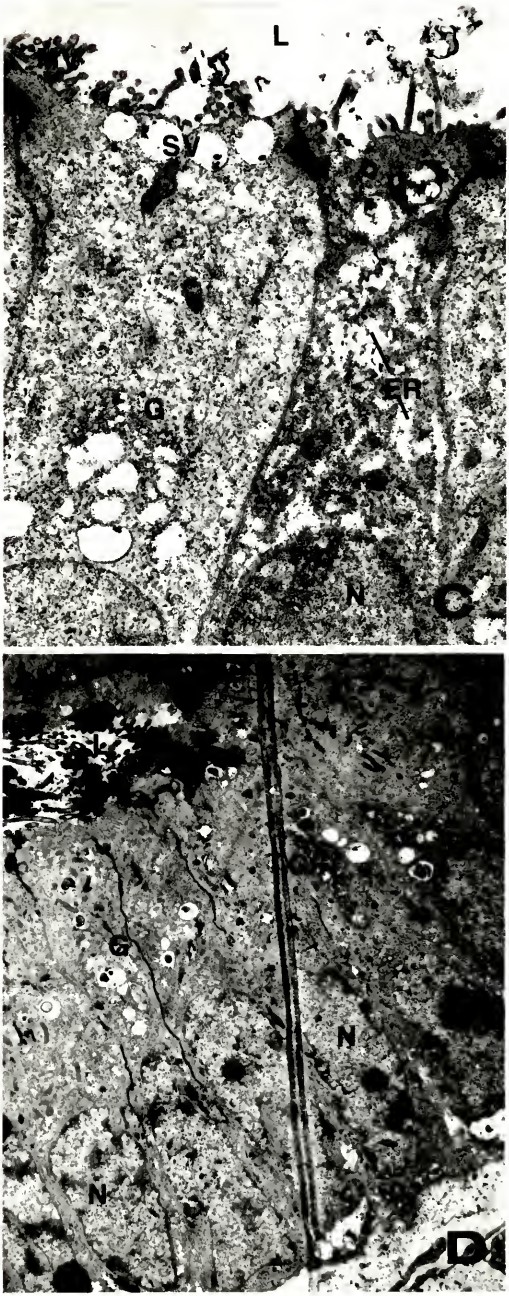
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BIOGRAPHICAL SKETCH

Randall Harrell Renegar was born to Lonnie Harrell and the late Clara Johnson Renegar on April 12, 1952, in Winston-Salem, North Carolina. He spent most of his childhood and adolescence in Charlotte, North Carolina, and graduate from Independence High School in 1970. He received Bachelor of Science degrees in animal science and zoology from North Carolina State University in December, 1974. While in undergraduate school, the author received a Moorman Fund Scholarship.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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